Universidade de Aveiro Departamento de Ciências Médicas

Ano 2019

INÊS FILIPA PEREIRA ABRUNHOSA AMARAL

Análise da expressão de lncRNAs em monócitos e osteoclastos de pacientes com artrite reumatóide

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação da Doutora Ana Gabriela Henriques, Investigadora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro, e co-orientação científica do Doutor Ângelo Miguel Silva Calado, Professor Auxiliar da Faculdade de Medicina da Universidade de Lisboa.

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SPR
SOCIEDADE PORTUGUESA DE REUMATOLOGIA

Dedico este trabalho aos meus pais. Obrigada por tudo.

o júri

presidente

Doutora Ana Margarida Domingos Tavares de Sousa
Professora Auxiliar Convidada do Departamento de Ciências Médicas da Universidade de Aveiro

Doutora Anita Raquel Quintal Gomes
Professora Adjunta da Escola Superior de Tecnologia de Saúde de Lisboa

Doutor Ângelo Miguel Silva Calado
Professor Auxiliar da Faculdade de Medicina da Universidade de Lisboa

.

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palavras-chave

Artrite reumatóide, erosão óssea, monócitos, osteoclastos, osteoclastogénese, RNAs longos não-codificantes (lncRNAs).

resumo

A artrite reumatóide (AR) é uma doença reumática crónica, inflamatória e imuno-mediada. A erosão óssea é uma das características da fisiopatologia da AR e está associada à gravidade da doença. As erosões ósseas em pacientes com AR resultam de um metabolismo ósseo alterado devido, em parte, a osteoclastogénese (o processo pelo qual as células precursoras da linhagem de monócitos se diferenciam em osteoclastos) e a atividade osteoclástica excessivas. Num estudo recente, observou-se uma expressão variável de RNAs longos não codificantes (lncRNAs) durante a osteoclastogénese no ratinho, sugerindo um papel fisiológico dos lncRNAs neste processo. Em pacientes com AR, vários estudos observaram até à data uma expressão alterada de lncRNAs em tipos celulares críticos da fisiopatologia da doença, como leucócitos mononucleares do sangue periférico e sinoviócitos ativados tipo-fibroblastos. No entanto, nenhum estudo examinou, até ao momento, a expressão de lncRNAs em monócitos e osteoclastos de pacientes com AR. Este trabalho visou abordar esta questão analisando a expressão de um painel de 8 lncRNAs (GAS5, NEAT1, Meg3, DANCR, HOTAIR, Meg9, H19 e Sox2OT) em monócitos e osteoclastos de pacientes com AR, artrite inicial e controlos saudáveis. Os doentes foram recrutados no Serviço de Reumatologia do Hospital de Santa Maria, Centro Académico de Medicina de Lisboa. Dadores emparelhados por idade e sexo foram usados como controlos saudáveis. Foi colhido sangue heparinizado, a partir do qual, se isolaram células mononucleadas de sangue periférico (PBMCs) por centrifugação em gradiente de densidade. Os monócitos aderentes foram depois diferenciados in vitro em osteoclastos por um protocolo de diferenciação de 21 dias. RNA total foi extraído de monócitos e osteoclastos, para posterior análise da expressão de lncRNAs por PCR quantitativo em tempo real (RT-qPCR). Estudou-se a expressão de lncRNAs para dezassete indivíduos, incluindo 7 pacientes com artrite reumatóide estabelecida, 3 pacientes com artrite inicial e 7 dadores saudáveis. Observou-se um número substancialmente superior de osteoclastos em pacientes com artrite inicial face ao obtido para controlos saudáveis. Observámos um aumento da expressão de NEAT1 e GAS5, e uma diminuição da expressão de DANCR, em monócitos de pacientes com AR estabelecida. Por outro lado, observou-se uma expressão aumentada de NEAT1 e diminuída de GAS5 e DANCR em osteoclastos de pacientes com artrite inicial. Não foram encontradas diferenças estatisticamente significativas. Os resultados obtidos são consistentes com um potencial osteoclastogénico aumentado em monócitos periféricos de pacientes com artrite inicial e, portanto, com um metabolismo ósseo alterado. Globalmente, os resultados de expressão de lncRNAs sugerem que os lncRNAs testados possam ter um papel na osteoclastogénese na AR, atendendo aos seus níveis de expressão alterados em monócitos de pacientes com artrite inicial e com AR.

keywords

Rheumatoid arthritis, bone erosion, monocytes, osteoclasts, osteoclastogenesis, long non-coding RNAs (lncRNAs).

abstract

Rheumatoid arthritis (RA) is a chronic inflammatory immune-mediated rheumatic disease. Bone erosion is one of the hallmarks of RA pathophysiology and is associated with disease severity. Bone erosions in RA patients result from an imbalanced bone metabolism due, in part, to excessive osteoclastogenesis (the process by which precursor cells of the monocyte lineage differentiate into osteoclasts) and osteoclastic activity. Variable expression of long non-coding RNAs (lncRNAs) has been observed during mouse osteoclastogenesis, suggesting a physiologic role for lncRNAs in the process. In RA patients, lncRNA expression has been further shown to be altered in cellular types critical for its pathophysiology, like peripheral blood mononuclear leukocytes and activated fibroblast-like synoviocytes, in comparison to healthy controls. However, no study has yet examined lncRNA expression in monocytes and osteoclasts of RA patients. This work aims to address this question by analyzing the expression of a panel of 8 lncRNAs (GAS5, NEAT1, Meg3, DANCR, HOTAIR, Meg9, H19, and Sox2OT) in monocytes and osteoclasts of early arthritis patients, established RA patients and healthy controls. Both groups of patients were recruited at the Rheumatology Department, Hospital de Santa Maria, Lisbon Academic Medical Centre, Portugal. Age and sex matched donors were used as healthy controls. Heparinized blood was collected from each participant. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples by density gradient centrifugation. Adherent monocytes were in vitro differentiated into osteoclasts by a 21-day differentiation protocol. Total RNA was extracted from both monocytes and osteoclasts, for further analysis of lncRNA expression by real-time quantitative PCR (RT-qPCR). LncRNA expression analysis was performed for seventeen subjects, including 7 established rheumatoid arthritis patients, 3 early arthritis patients and 7 healthy donors. In vitro osteoclastogenesis produced a substantially higher number of osteoclasts in early arthritis patients, when compared to healthy controls. From our lncRNA panel, only GAS5, NEAT1, and DANCR presented a measurable expression in all tested samples. Our results showed an increased expression of NEAT1 and GAS5 along with a decreased expression of DANCR in monocytes of established RA patients, in comparison to those of healthy controls. An increased expression of NEAT1 along with a decreased expression of GAS5 and DANCR was observed in osteoclasts of early arthritis patients, when compared to those of healthy controls. No statistically significant differences were found for both analyses. Our data are consistent with an increased osteoclastogenic potential of peripheral monocytes of early arthritis patients, and thus, with an imbalanced bone metabolism in this pathological condition. Overall, our results prompted us to suggest that the lncRNAs here analyzed may in fact play a role in osteoclastogenesis in RA, as an altered lncRNA expression was observed in monocytes of early arthritis and established RA patients, when compared to healthy controls.

POSTER PRESENTATIONS UNDER THE SCOPE OF THIS THESIS

"Probing the expression of lncRNAs in monocytes and osteoclasts of rheumatoid arthritis patients", Inês Abrunhosa Amaral, Ângelo Calado, Vânia G. da Glória, Rui L. Teixeira, Sofia Barreira, João E. Fonseca. Poster presented at XXI Congresso Português de Reumatologia, p123, **2019**, Vilamoura, Portugal.

"Analyzing the expression of lncRNAs in monocytes and osteoclasts of rheumatoid arthritis patients", Inês Abrunhosa Amaral, Ângelo Calado, Vânia G. da Glória, Rui L. Teixeira, Sofia Barreira, João E. Fonseca. Poster presented at V Post-graduate Symposium in Biomedicine, p22, **2019**, Aveiro, Portugal.

LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
ADA	Adalimumab
anti-CCP	Anti-cyclic citrullinated peptide
APC	Antigen-presenting cell
BM	Bone marrow
BMU	Basic multicellular unit
BMU	Basic multicellular unit
CCP	Cyclic citrullinated peptide
CCR6	C-C chemokine receptor type 6
COX	Cyclooxygenase
CRP	C-reactive protein
CTLA4	Cytotoxic T-lymphocyte–associated antigen 4
CZP	Certolizumab pegol
DANCR	Differentiation antagonizing non-protein coding RNA
DAS28	Disease activity score based on 28 joints
DCs	Dendritic cells
DMARD	Disease-modifying antirheumatic drug
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
ENCODE	Encyclopedia of DNA Elements
ESR	Erythrocyte sedimentation rate
ETC	Etanercept
EULAR	European league against rheumatism
FBS	Fetal bovine serum
FLS	Fibroblast-like synoviocyte
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS5	Growth arrest specific 5
GLM	Golimumab

LIST OF ABBREVIATIONS (continuation)

GRE	Glucocorticoid response element
GWAS	Genome-wide association studies
H19	H19 imprinted maternally expressed transcript
HLA	Human leukocyte antigen
HOX	Transcript antisense RNA
HSCs	Hematopoietic stem cells
IFNγ	Interferon γ
IFX	Infliximab
IGF	Insulin-like growth factor
IL	Interleukin
IL23R	Interleukin 23 receptor
IRF5	Interferon regulatory factor 5
lncRNA	Long non-coding RNA
LPS	Lipopolysaccharides
MCSF	Macrophage colony stimulating factor
MEG3	Maternally expressed 3
MEG9	Maternally expressed 9
MHC	Major histocompatibility complex
miRNA	Microrna
MMP	Matrix metalloproteinase
MO	Monocyte
MTX	Methotrexate
ncRNA	Non-coding RNA
NEAT1	Nuclear paraspeckle assembly transcript 1
NSAID	Non-steroidal anti-inflammatory drug
OC	Osteoclast
OPG	Osteoprotegerin
OPGL	Osteoprotegerin ligand

LIST OF ABBREVIATIONS (continuation)

PADI4	Peptidyl arginine deiminase type IV
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PGs	Prostaglandins
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RA	Rheumatoid arthritis
RAF1	Raf-1 proto-oncogene, serine/threonine kinase
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RF	Rheumatoid factor
RF	Rheumatoid factor
RNA	Ribonucleic acid
sncRNA	Small non-coding RNA
SNPs	Single-nucleotide polymorphisms
snRNA	Small nuclear RNA
Sox2ot	SOX2 overlapping transcript (non-protein coding)
STAT4	Signal transducer and activator of transcription 4
Th1	T helper 1
Th17	T helper 17
TNFα	Tumor necrosis factor alpha
TRAP	Tartrate-resistant acid phosphatase

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INTRODUCTION

INTRODUCTION

1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory and immune-mediated disease [1,2]. It is an idiopathic disease (of unknown cause), that can be defined as a clinical syndrome spanning several disease subsets, with multiple pathogenic mechanisms [1–3]. The hallmark of RA is systemic inflammation, with persistent symmetric peripheral polyarthritis and structural damage of cartilage and bone tissue, leading to joint swelling, loss of function and stiffness [2]. The hypersensitivity reaction associated with disease pathogenesis in RA is mediated by immune complexes (type III reaction), and the main autoantibodies involved are rheumatoid factor (RF) and anti-citrullinated protein antibody (anti-ACPA) [4,5]. RA patients can be subdivided into two subtypes designated "seropositive" and "seronegative", with seropositivity being defined as the presence of serum elevations of the autoantibodies RF and/or ACPAs [6].

The pain and progressive deterioration of joint function, along with increased comorbidity, have a high impact on health and quality of life of RA patients. In severe cases, the ability to work can be compromised [7]. Different criteria can be applied to assess disease severity in RA: disease activity, extraarticular manifestations and joint destruction [8]. Concerning this, the Disease Activity Score based on 28 joints (DAS28) is one of the most widely used tools to assess disease activity in RA patients [9].

1.1. Epidemiology

Worldwide prevalence of RA is of around 1%, with a significant variation of the disease incidence and prevalence among different populations [10]. In Portugal, the total prevalence of RA is of 0.7%, with a four-fold higher prevalence in women than in men (1.2% and 0.3% respectively) [11]. It is the most common form of chronic inflammatory arthritis [12]. A systematic review by Minichiello *et al*., described a trend toward a decline in the incidence of RA [8]. However, while some studies support such decrement [13,14], others report an increasing trend in the incidence of RA in the general population [15,16].

Patients with rheumatoid arthritis present a life expectancy decreased by 3 to 10 years when compared with the general population, along with a 50%-54% increased risk of premature mortality [7]. Higher severity of the disease is associated with a higher risk of death [7]. Cardiovascular

diseases, respiratory diseases, and infections are among the primary causes of death in RA patients [7,17]. The increased risk for infection in RA patients may be attributed to the impaired immune function in RA or an effect of immunosuppressive therapy. Death by an infection in RA patients is often due to respiratory infection or pneumonia [17]. Recent trends show no significant reduction in mortality in RA patients worldwide [7]. However, information about the mortality rates of observational studies differ. Some studies show higher mortality in RA patients [18,19], while others report that mortality in patients with RA was not different from that of the general population [20,21].

Several studies show a decrement in disease activity over time, based on a decline of DAS28 values, suggesting a decrease in RA severity in most recent cohorts [22–24]. Regarding overall extraarticular manifestations, there is no consensus. Some authors report a decrease [25], while others describe no significant changes over time [26]. A study from the UK, in a stable well-defined population, monitored for 15 years, reported a decrease in the incidence of systemic rheumatoid vasculitis [27]. On the other hand, the need for joint surgery reflects the degree of joint destruction [8]. Increased severity of disease leads to an increased need for joint surgery. Some studies report a decline in the severity of joint destruction, in RA patients, over time [28,29]. Regarding the need for joint surgery, several authors indicate a significant reduction in both the risk and the incidence of joint surgery [30–32].

These varying results might be explained by differences in treatment or different types of cohorts and follow-up time [33]. Nevertheless, evidence indicates a decrease in severity over time, with fewer extraarticular manifestations and lower disease activity, along with a diminished need for surgery to treat joint destruction, and less severe radiological changes [8]. Van Nies *et al*., reported an improvement in the outcome of early RA patients over the last decade, based on lower rates of joint destruction and higher remission rates [20]. The authors hypothesized that current treatment strategies, namely the use of disease modifying anti-rheumatic drugs (DMARDs), combination therapies and tight control of disease activity, have been having a positive impact on the survival of RA patients, thus reflected in a reduction of the mortality risk in RA [20].

1.2 Characteristics and Pathophysiology

Rheumatoid arthritis comprises different disease subsets that involve several immuno-inflammatory cascades, with predominant features varying from patient to patient [2]. Nevertheless, all the involved cascades in RA lead towards a final common pathway characterized

by persistent synovial inflammation (hyperplasia) and associated damage to articular cartilage and underlying bone tissue [2]. The characteristic hyperplastic joint present in RA, with several inflammatory cellular infiltrations, is represented in **Figure 1**.

Even though joint involvement is the hallmark of this disease, RA is an organ nonspecific (systemic) immune-mediated disease since extraarticular pathologic lesions or manifestations can be found, including fatigue, subcutaneous nodules, lung involvement, pericarditis, peripheral neuropathy, vasculitis, and hematologic abnormalities [12].

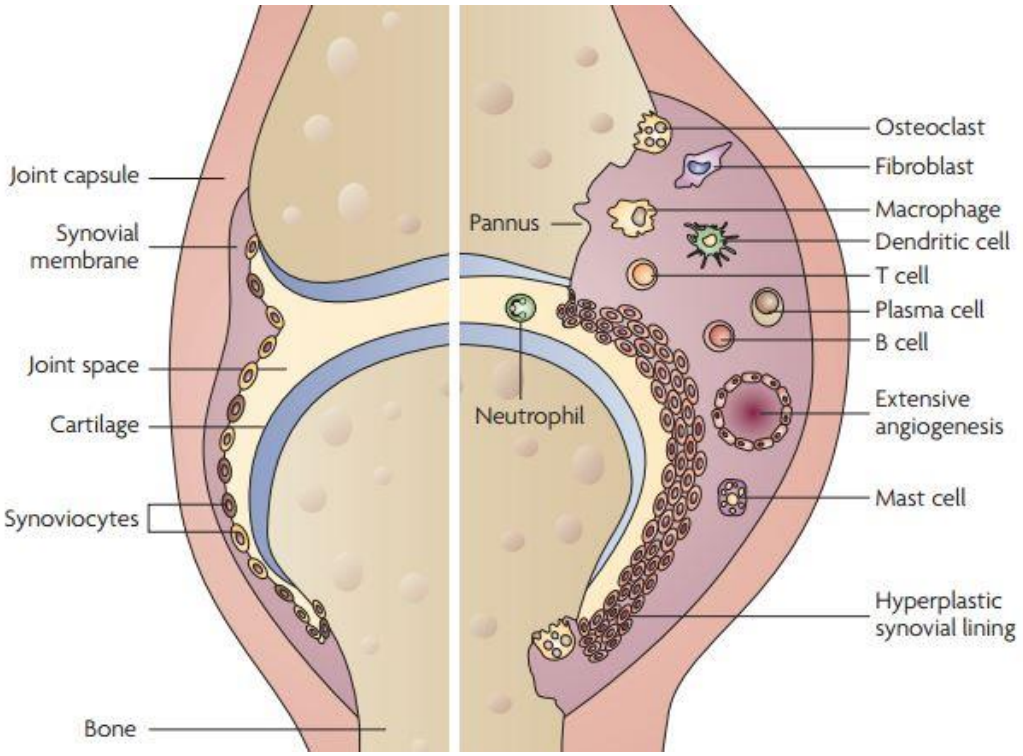

Figure 1 - Cartoon representation of a normal joint (left) and a rheumatoid arthritis joint (right). Adapted from Strand *et al.,* 2007 [265].

The pathophysiological mechanisms that have been proposed for the development of RA involve a complex interplay between different cells, genetic and environmental factors, triggers, and chance [34]. Both T and B cells and the action of pro-inflammatory cytokines play key roles in the pathophysiology of RA [35]. Tumor necrosis factor alpha (TNF-α) and interleukin (IL-6) are regarded as the cytokines most directly implicated in RA, although IL-1 and IL-17 may also play an important role in the disease process [35–37]. Prominent T-cell infiltrate in RA patients suggests these cells

are key participants in the acute inflammatory features of RA [1]. Nevertheless, increasing evidence suggests that a variety of innate effector cells, such as monocytes and macrophages, are also closely involved in the development of synovial inflammation in RA. In fact, massive infiltration of activated monocytes/macrophages are frequently observed in synovial membranes of RA patients. These are a major source of cytokines (such as TNF-α, IL-1β, IL-8, and GM-CSF) in the inflamed joints [38].

The initial milestone for RA pathogenesis onset is thought to be the activation of the innate immune response, including the activation of dendritic cells by exogenous material and autologous antigens [35]. Antigen-presenting cells, including dendritic cells, macrophages and activated B cells, present arthritis-associated antigens to T cells [35,39]. These T cells are upregulated by various lymphokines, including interleukin 2 (IL-2) and interferon γ (IFNγ). Upon stimulation, T cells induce activation of macrophages, B cells, fibroblasts, and osteoclasts [38,39]. In turn, B lymphocytes express various cell-surface molecules, including antigen receptor immunoglobulin and differentiation antigens, such as CD20 and CD22. Upon differentiation, they turn into plasma cells that secrete antibodies, including autoantibodies to IgG (rheumatoid factor), to citrullinated peptides such as vimentin, fibrinogen, or cyclic citrullinated peptide (CCP) and to rheumatoid arthritis antigen (RA33) [42,43]. On the other hand, the formation of immune complexes by autoantibodies can increase the production of proinflammatory cytokines such as TNF-α. In fact, the occurrence of autoantibodies RF and anti-CCP are associated with severe rheumatoid arthritis and can be used as an effective strategy for identifying patients with RA at high risk for poor outcome [44]. When activated, B cells also serve as APCs, inducing T cell activation, and potentially leading to the perpetuation of the immune-mediated proinflammatory response [42]. Besides cells of the innate and adaptive immune system, several other inflammatory cell populations infiltrate the synovial membrane in rheumatoid arthritis patients [34,35,45].

The *in situ* pathological occurrences within the synovial inflammation include activation of endothelial cells, along with neovascularization and increased presence of fibroblast-like synoviocytes (FLSs). These synoviocytes are highly activated and secrete inflammatory mediators, cytokines and matrix metalloproteinases (MMPs) [46,47]. MMPs are enzymes that can degrade the components of the extracellular matrix of the joint [48]. By secreting MMPs into the synovial fluid, and probably by direct invasion, fibroblast-like synoviocytes can destroy cartilage and play a role in bone destruction [46,49]. Nevertheless, bone erosions occur mainly via activated osteoclasts. Bone erosions in RA patients result from an imbalanced bone metabolism due, in part, to excessive osteoclastogenesis and osteoclastic activity, that leads to increased bone resorption by osteoclasts, and inadequate bone formation by osteoblasts [50–52]. Besides being responsible for bone

resorption, osteoclasts are key players in the complex crosstalk between bone and immune cells, that leads to the perpetuation of the inflammatory environment in joints of RA patients [53–55]. The role of osteoclasts in physiological bone metabolism and in RA pathogenesis will be discussed further ahead.

All the above-mentioned events and complex cross-talk of immune and inflammatory modulators promote influx, expansion, and activation of cells in the synovium, ultimately leading to the immunoinflammatory and destructive response of rheumatoid arthritis, clinically presented as joint swelling and joint and bone destruction [56,57]. However, the precise hierarchy of events remains enigmatic. It is nowadays understood that multiple and redundant pathophysiological mechanisms contribute to RA [56,57].

1.3 Diagnostic and Treatment

Diagnostic of RA is mostly clinical, along with a combination of laboratory tests, and imaging methods. The most recent criteria to the diagnosis of rheumatoid arthritis are the 2010 American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) criteria [58], and their application is due to be made by the physician regarding each patient's individual circumstances. These guidelines are aimed at identifying rheumatoid arthritis among patients that recently present synovitis (inflammation of a synovial membrane) in at least one joint, in the absence of an alternative diagnosis that better explains the synovitis. For patients to be diagnosed with RA, they need to achieve a total score of at least 6 (of a possible 10) within 4 evaluation domains [58,59]. These domains include the following: (1) the number and site of involved joints, (2) serologic abnormalities (presence of rheumatoid factor or anti-citrullinated peptide/protein antibody), (3) elevations of inflammatory markers (erythrocyte sedimentation rate and/or C-reactive protein [CRP]), and (4) the duration of symptoms [58]. The criteria for the evaluation of disease progression are the 2012 ACR Disease Activity Measures [60].

To treat RA patients, there are multiple drugs currently available for use. These can be non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs) or adjunctive agents such as corticosteroids, and analgesics [61–63]. Most of the NSAIDs are designed to target and suppress prostaglandins (PGs), inflammatory mediators, through inhibition of the cyclooxygenase (COX) enzymes [61]. These can lead to renal, hepatic, and cardiovascular toxicity with long-term use in RA patients [61]. Within the NSAIDs, there are many different drugs, such as Ibuprofen, Aspirin, and Naproxone [61].

DMARDs are a class of antirheumatic drugs which can be divided into two sub-categories: non-biological (synthetic) and biological [62]. Among non-biological DMARDs, methotrexate (MTX) has been the gold standard for the past few decades in the treatment of RA patients [62,63]. Although its exact mechanism has not been fully defined, MTX can block cytokine production and modulate specific matrix metalloproteinases (MMPs) levels [64,65].

The introduction of biological DMARDs (bDMARDs) dramatically changed the treatment and outcome of RA, as they are significantly more effective than non-biologic treatments in improving physical function in RA patients [66]. These drugs include monoclonal antibodies targeting tumor necrosis factor (TNF)-α (anti-TNF-α), IL-6 receptor (anti-IL6R), IL-1 receptor (anti-IL1R) and other inhibitors of cytokines and immune cells that play a major role in promoting RA pathogenesis [67]. As previously described, cytokines play a fundamental role in the pro-inflammatory milieu occurring within the synovium of affected joints in RA patients [36,68]. Among these, TNF-α and IL-6 are considered the most relevant and constitute the therapeutic targets of several drugs for rheumatoid arthritis [69]. These drugs can directly inhibit these cytokines, or interfere with receptor binding, or both. Up to date, a total of five anti-TNF drugs have entered clinical use, namely infliximab (IFX), adalimumab (ADA), etanercept (ETC), golimumab (GLM), and certolizumab pegol (CZP) [70]. Infliximab (IFX) was the first TNF-α inhibitor to be administered in RA patients. Both infliximab, adalimumab, and golimumab are monoclonal antibodies anti-TNF-α. Certolizumab pegol is a humanized monovalent Fab antibody fragment linked to polyethylene glycol (PEG) [70]. Etanercept was the first biological DMARD to be approved for treating RA and is a recombinant fusion protein in which the extracellular domain of TNF receptor II is fused to the Fc-portion of IgG1 [70,71]. IL-6 has also been the focus of great interest in RA. This cytokine in presumed to play key roles in the context of the pathogenesis of rheumatoid arthritis: activation of T cells, macrophages and osteoclasts, and induction of antibody production by B cells [37,72–74]. Tocilizumab is a monoclonal antibody to the IL- 6 receptor, that targets the actions of this cytokine [75,76].

However, despite the range of available treatments, several unmet needs remain. There is a lack of predictive biomarkers of prognosis, therapeutic response, and toxicity. Importantly, current synthetic and biological DMARDs therapies sometimes fail or produce only partial responses. In addition, sustained remission in RA patients is rarely achieved and requires ongoing pharmacologic therapy [77].

1.4 Risk Factors

Multiple genetic and environmental factors have been associated with an increased risk of developing rheumatoid arthritis [78]. In RA, altered gene expression is present in both circulating immune cells and synovial tissues [79].

The genetic factors can include susceptibility genes such as HLA-DRB1, which shows the strongest association with RA, in particular with ACPA positive RA [80]. Most HLA DRB1 alleles that predispose to RA (HLA DRB1*01, *04, and *10 alleles) share a specific amino acid sequence in their peptide-binding region. This sequence similarity might implicate the involvement of these shared epitope alleles in presenting arthritis-related peptides. Some studies have reported that these alleles may preferably present citrullinated peptides, which could explain the strong association with ACPA positive RA [81,82]. RA patients expressing two HLA-DRB1*04 alleles are at elevated risk for nodular disease, major organ involvement and increased need for surgery related to joint destruction [83]. Furthermore, HLA and non-HLA genes may discriminate anti-cyclic citrullinated peptide (anti-CCP) antibody–positive and anti–CCP–negative RA groups [84].

Genome-wide association studies (GWAS) have expanded the number of validated RA risk loci, identifying additional alleles related to RA risk beyond HLA-DRB1 shared epitope alleles [84]. Single-nucleotide polymorphism genotyping across the MHC has identified additional alleles related to RA risk, including those found on the conserved A1-B8-DR3 (8.1) haplotype and those near the HLA-DRB1 gene. Some of the relevant single-nucleotide polymorphisms (SNPs) linked to RA pathogenesis include RAF1, STAT4, CTLA4, IRF5, CCR6, PTPN22, IL23R, and PADI4 [85–87]. Among these, some of the most relevant include PTPN22 and PADI4 [86,87]. Like HLA-DRB1, PTPN22 is mostly associated with ACPA positive RA. It encodes a tyrosine phosphatase involved in T and B cell signaling [87]. PADI4 encodes for an arginine deiminase responsible for the posttranslational conversion of arginine into citrulline residues. Besides being associated with RA susceptibility, the PADI4 haplotype is also mainly associated with ACPA positive RA [86].

Among the multiple environmental, lifestyle, and behavioral risk factors already studied, cigarette smoking is regarded as the most strongly and consistently associated with the development of RA, presenting a clear dose-response [88–90]. As well as the susceptibility genes, environmental factors such as smoking are most strongly associated with the seropositive RA phenotype [91]. It is estimated that smoking is responsible for around 35% ACPA positive RA cases. Furthermore, 55% of ACPA positive RA can be attributable to smoking, in individuals carrying two copies of the HLA-DRB1 shared epitope [91].

2. Monocytes

Human monocytes represent 5 % – 10% of the blood leucocytes and their half-life in the vascular compartment is around 1 – 3 days. Monocytes in blood have some typical morphological features such as the irregular shape of the cell and its nucleus, high cytoplasm-to-nucleus ratio, and light blue cytoplasm [92–94]. These cells originate in the bone marrow from hematopoietic stem cells (HSCs). They are then released into the peripheral blood, where they circulate. These circulating monocytes give rise to tissue-resident macrophages and specialized cells, such as dendritic cells (DCs) and osteoclasts [93]. Monocytes have important roles in both innate and adaptive immunity, primarily functioning in immune defense, inflammation, and tissue remodeling, in both homeostasis and inflammation. During inflammation, monocytes circulate through the blood and extravasate into inflamed tissues, where they differentiate into macrophages, generally maintaining the same inflammatory phenotype [95].

Monocytes are categorized into three major subsets, according to the expression of CD14 (the LPS co-receptor) and CD16 (the low-affinity receptor for the Fc region of IgG): classical $CD14^{++}CD16^{-}$ that constitute about 90% of circulating monocytes, and intermediate $CD14^{++}CD16^{+}$ and non-classical $CD14^{+}CD16^{+}$ that together constitute the remaining 10% [96,97]. The first two populations of monocytes share many features, including a high capacity for phagocytosis and proinflammatory cytokine production. Classical monocytes, the dominant blood population, express CCR2, a receptor involved in mobilization from the bone marrow (BM) and recruitment to inflammatory sites, which is the main function of classical monocytes [98,99]. In contrast, non-classical monocytes present a reduced CCR2 but elevated CX3CR1 surface expression, which is needed for patrolling blood vessels and migrating into resting tissues [96,100]. The population of intermediate monocytes expresses both CCR2 and CX3CR1 on intermediate level and shows the highest HLA-DR expression [101].

2.1. Role of monocytes in the pathophysiology of rheumatoid arthritis

Infiltration of monocytes along with T and B cells and the exacerbated presence and activity of macrophages and osteoclasts into the synovial tissue and production of inflammatory mediators typify the immunopathology of RA [102]. These accumulating activated monocytes massively infiltrate inflammatory sites (synovial membranes) of RA patients and present a significantly increased expression of CD16 on their surface and produce TNF-α in response to TLR stimulation [103–107]. The impact of the monocytic lineage in determining the immune response is substantial and these cells interfere with both the innate and adaptive immunity [108]. For example, in RA,

monocytes present in inflamed joints have been shown to induce the development of Th17 cells, thus contributing to the amplification of inflammation. In fact, T cell - monocyte contact is involved in cartilage destruction and cytokine production [109,110]. Therefore, targeting monocyte-derived cytokines such as tumour necrosis factor (TNF), IL-1, IL-6 or even monocyte - T cell interaction might be a therapeutic strategy for controlling inflammation in RA patients non-responsive to classical DMARDs [111]. Approaches to depletion of monocytes using specific antibodies could prevent their presence in the synovial tissue and thus, attenuate inflammation [112,113].

In RA patients, the frequencies of classical, intermediate and non-classical blood monocytes are altered, when compared to healthy controls [114–116]. $CD14^{+}CD16^{+}$ monocytes (non-classical) are increased in the blood of RA patients, which might contribute to the persistent joint inflammation of RA [117]. These monocytes display properties for antigen presentation and inflammatory characteristics upon activation [118]. Increased expression of CD16 in monocytes was observed in patients with active disease and increased counts of tender and swollen joints. CD16 expression on RA blood monocytes was augmented in vitro by IL-10, M-CSF, and TGF-β_1 [117]. These pieces of evidence suggest that monocytes are key players in RA pathogenesis, contributing the perpetuation of the inflammatory milieu observed in the joints of RA patients. However, it is still unknown whether and how the altered frequency of monocytes subsets in RA patients affects the role of monocytes and/or macrophages in the pathogenesis of RA.

In addition to their central role in modeling inflammation, monocytes can be pinpointed as the origin of pathological bone erosion in RA, due to their excessive differentiation into osteoclasts [119]. As such, monocytes may thus constitute ideal targets to modulate osteoclastogenesis in inflammation and RA.

3. Osteoclasts

An osteoclast can be described as a tissue-specific macrophage of the bone, able to degrade bone tissue by polarized secretion of proteolytic enzymes such as cathepsin K and acid, which hydrolyzes and solubilize the organic and inorganic components of bone [50,120]. In fact, in physiological conditions, the osteoclast is the only cell type capable of resorbing bone [120]. Mature osteoclasts are fused polykaryons (multinuclear cells) arising from multiple (up to 10–20) individual cells [50].

Osteoclastogenesis is the process by which hematopoietic precursor cells of the monocyte/macrophage lineage differentiate into osteoclasts [50,120]. In particular, peripheral blood monocytes (PBMs) constitute the main source of precursors of osteoclasts, like as with other

tissue macrophages [121,122]. PBMs leave the peripheral circulation by margination in capillaries and migration into the extravascular pool. Osteoclasts arise from these migrating monocytes by in situ proliferation of macrophage precursors and further differentiation in tissues [93]. For such processes, two major cytokines are required: the macrophage colony stimulating factor (MCSF) and the receptor activator of NF-kB ligand (RANKL) supplied mostly by osteoblasts and/or osteocytes [50,120]. M-CSF binds to its receptor c-FMS, (cellular-feline McDonough strain sarcoma virus oncogene homologue, or CSF-1 receptor, or CD115), which in turn induces the expression of RANK on monocytes. RANKL expression by synovial fibroblasts is induced by pro-inflammatory cytokines such as TNF-a, IL-1, IL-6, and IL-17 [123]. The RANK/RANKL ligand-receptor complex is a crucial intervenient in osteoclast differentiation and activation. RANKL is a necessary factor for the differentiation of osteoclasts and functions as both survival factor and activator [124]. Its physiological inhibitor is osteoprotegerin (OPG) [124]. Osteoblasts are the main source of RANKL. However, it can be expressed by synovial cells, activated T cells, mature B cells and NK cells. RANKL is a mediator of differentiation, survival, and activation of osteoclasts and its expression is upregulated by TNF, IL-1, IL-6, and IL-17 [124]. Overall, osteoclastogenesis and osteoclast activation are regulated, both positively and negatively, by at least 24 gene or loci [50]. Among these are as well cytokines, produced by monocytes, with important effects on osteoclast differentiation, activation, and apoptosis [121].

3.1. Role of osteoclasts in the pathophysiology of rheumatoid arthritis

Osteoclasts play a critical role in skeletal homeostasis by regulating bone resorption. However, in RA, they excessively populate chronically inflamed joints, resorbing the bone in an exacerbated way and creating localized skeletal defects, the so-called bone erosions [125]. Chronic inflammation appears to be a key mediator for both local and systemic bone loss in RA patients [124]. In fact, inflammation itself might trigger bone resorption by osteoclasts [126]. It has been reported that the high levels of pro-inflammatory cytokines prevalent in the synovial fluid of affected joints in RA patients are associated with osteoclast-mediated focal bone erosion at the margins of these joints [127]. Pro-inflammatory cytokines are potent mediators of bone loss and correlate with greater disease activity in RA patients. They can enhance osteoclastogenesis in inflamed joint and bone, acting on osteoclasts, osteoblasts, and their precursors, by inducing RANKL expression [124,128,129].

RA patients present increased numbers of osteoclast progenitors in the peripheral blood, exhibiting an enhanced ability to differentiate into osteoclasts when compared to those of matched healthy controls [130]. The extensive infiltration of monocytes in the synovial tissue of inflamed joints in RA patients leads to an increased number of osteoclasts, as these function as osteoclast precursors and provide specific molecular signals that drive osteoclast formation, as previously stated [131]. Overall, osteoclasts play a critical role in bone destruction in RA and could represent a therapeutic target for RA [132].

The role of monocytes and osteoclasts in the pathophysiology of rheumatoid arthritis is represented in **Figure 2**.

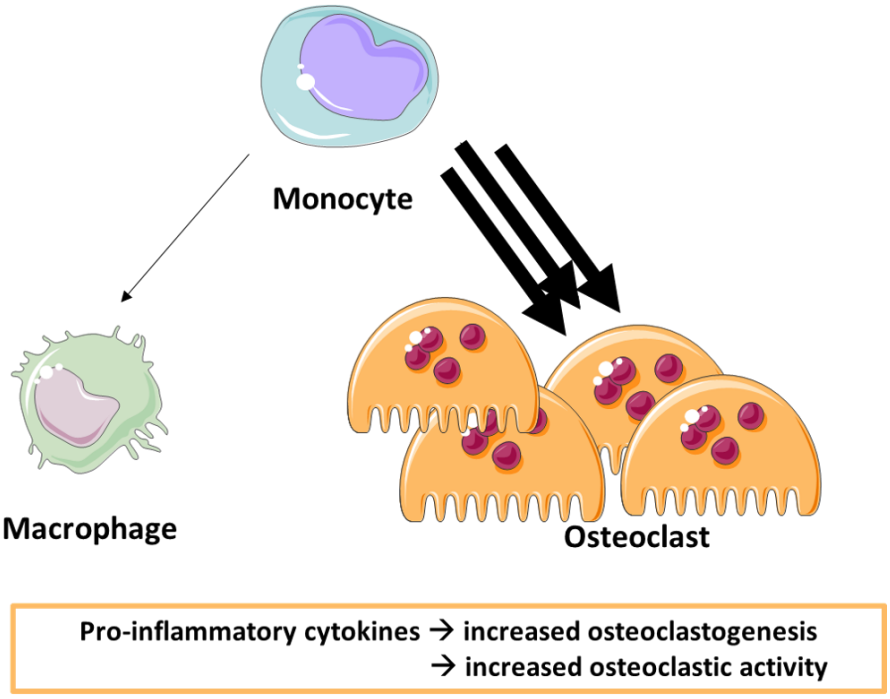

Figure 2 - Cartoon representation of the role of monocytes and osteoclasts in RA pathogenesis.

4. Bone metabolism

Bone homeostasis is modulated by both inflammation and immunological events. The concept of osteoimmunology was coined a few years ago to account for the interplay between the bone and immune systems [123,133]. During life, bone undergoes modeling and remodeling to grow or change shape. The main cells involved in bone remodeling are osteoblasts and osteoclasts, whose

functions are tightly coupled. Additional cells involved in bone metabolism are osteocytes and the bone lining cells [134].

The osteoblast is a specialized bone-forming cell derived from mesenchymal stem cells (MSCs). Osteoblasts produce bone by synthesis and secretion of type I collagen, that makes up the major bone matrix protein. They also participate in the mineralization of the newly formed bone [135]. As previously stated, osteoclasts are the cells capable of resorbing bone. For osteoclasts to be activated and bone resorption to commence, non-polarized, mature osteoclasts must bind to the bone matrix. When osteoclasts bind to bone, they become polarized [120]. Osteocytes are the most abundant and long-lived cells in bone. They comprise 90-95% of the total bone cells and have a lifespan of up to 25 years. Osteocytes originate from osteoblasts that have undergone terminal differentiation during bone formation and subsequently have been engulfed by the unmineralized osteoid. Following mineralization, these entombed cells become osteocytes [136]. Its functions include monitoring bone quality and stress, as well as coordinate remodeling through membrane-bound and secreted factors [120,137]. Bone lining cells are quiescent flat-shaped osteoblasts that cover the bone surfaces, where neither bone resorption or bone formation occurs [138].

4.1. Normal bone metabolism: modeling and remodeling

Bone is a complex tissue, undergoing continuous remodeling to heal damage, grow and maintain calcium and phosphate homeostasis [120,139,140]. The human bone is composed of trabecular bone, the fine bony network hosting the bone marrow, and cortical bone, the dense bony shell that provides structural support in weight-bearing regions [51]. A healthy bone metabolism relies on a delicate balance between bone formation by osteoblasts and bone resorption by osteoclasts [120,139,140]. The concerted and sequential actions of both osteoclasts and osteoblasts in the process of bone remodeling has been termed the basic multicellular unit (BMU) [139–141].

It is important to note that bone remodeling differs from bone modeling. Bone modeling is the process by which the generation and maintenance of the shape of bone during skeletal growth occur, allowing bones to change shape or size in accordance to physiologic influences or mechanical forces [142,143]. This process lasts from the beginning of skeletal development in fetal life until the end of the second decade of life, which is when the longitudinal growth of the skeleton is completed [140]. In turn, bone remodeling is a continuous process, occurring throughout life, with the purpose of maintaining strength and mineral homeostasis in the bone tissue [144]. Osteoclastic resorption

is tightly coupled to the osteoblastic bone formation during the bone remodeling cycle [139,145,146].

The remodeling occurs within the BMU and is a process defined by five phases: activation, resorption, reversal, formation, and termination [140,146]. The bone lining cells separate from the underlying bone and form a raised cover that encases each BMU, exposing the bone surface at the site to be remodeled and creating a unique environment for coupled resorption–formation [147]. Each active BMU consists of bone-resorbing osteoclasts covering the newly exposed bone surface, resorbing the old bone and preparing the surface for the deposition of replacement bone. Following immediately behind, the osteoblasts secrete and deposit unmineralized bone osteoid [148,149]. The orderly arrangement of the cells within the BMU is critical for correct remodeling, ensuring the correct sequence of the phases of the process [148,149].

The first phase of the bone remodeling cycle is the activation phase, which is characterized by the recruitment of osteoclasts [140,146]. The initiating signal for remodeling can be either hormonal or mechanic and is supposed to be detected by the osteocytes. In fact, osteocytes are key players in the bone remodeling cycle modulation [137,150,151]. Succeeding the detection of the signal occurs the retraction of the bone lining cells and the digestion of the endosteal membrane by collagenase. Alongside with this, occurs the recruitment of precursor cells from the monocyte/macrophage hematopoietic lineage, which are recruited from circulation and are activated at the site [152–154]. Multiple mononuclear cells fuse to form multinucleated preosteoclasts that migrate and attach to the bone surface. These develop and terminally differentiate in activated osteoclasts after binding to the bone matrix and thus form bone-resorbing compartments, secreting acid and lytic enzymes to break down the bone matrix [50,155].

The second phase of the bone remodeling cycle is the resorption phase when the osteoclasts resorb bone. This phase lasts approximately two to four weeks during each remodeling cycle [140,146]. Osteoclasts generate an acidic microenvironment in the attachment zone, secreting hydrogen ions that drop the pH as low as 4.5 within the bone-resorbing compartment [156]. These acidic conditions might help mobilize bone mineral and could favor the action of secreted lysosomal proteinases in the degradation of collagen [156–158]. The degradation of the collagen-rich bone matrix by osteoclasts occurs through the secretion of specialized proteinases, such as matrix metalloproteinases (MMPs) and cathepsin K [157,159]. At last, to ensure that excess resorption does not occur, the resorption phase is terminated by osteoclasts programmed cell death [160].

The third phase of the bone remodeling cycle is the reversal phase, where the osteoclasts undergo apoptosis and the osteoblasts are recruited. This phase lasts approximately four to five weeks in

each bone remodeling cycle [140,146]. Reversal phase is characterized by a shift from bone resorption to bone formation when osteoclasts are replaced by osteoblast-lineage cells which initiate bone formation [139,145]. However, the multitude of coupling signals between osteoclasts and osteoblasts that lead to this phase is not yet fully understood. Current understanding suggests that the release of osteogenic signals from the osteoclasts might initiate the coupling activity of the reversal phase [161,162]. There is evidence that osteoclasts (OCs) can produce many different molecules that affect the osteoblast lineage, both positively and negatively. These include OC-derived cell surface signals, OC-derived soluble signals and resorption-derived soluble signals [162]. Cells of the osteoblast lineage also appear to play an essential role in the concerted effort in bone remodeling, regulating bone-resorbing activity by osteoclasts and coupling resorption with the formation during the reversal phase [163]. These regulatory signals and coupling factors, produced by either osteoclasts or cells from the osteoblast lineage, can be cytokines and/or regulatory receptors on the cells surface, such as IL-6, BMP-2, TGF-β, insulin-like growth factor (IGF), prostaglandin E2 (PGE_2), receptor activator of NF-kB (RANK)/RANK ligand (RANKL) and osteoprotegerin (OPG)/OPG ligand (OPGL), functioning for instance in the ephrin-Eph bidirectional signaling between osteoblasts and osteoclasts [163–167]. Summarizing, the recently resorbed bone surface is prepared for the deposition of new bone matrix, and complex signaling occurs to couple resorption to bone formation, leading to the replacement of osteoclasts by osteoblast-lineage cells which initiate bone formation [161].

The fourth phase of the bone remodeling cycle is the formation phase when new bone formation occurs. This phase can last approximately up to four to six months [140,146]. Osteoblasts synthesize new bone matrix (osteoid), rich in type I collagen, filling the cavities left behind by osteoclastic bone resorption [168]. As the new bone matrix is gradually mineralized it gives origin to new bone. This process is called bone mineralization, whereby hydroxyapatite crystals are deposited among collagen fibrils. This process is regulated by controlling systemic levels of calcium and phosphate concentrations, along with the local concentration of calcium and phosphate within extracellular matrix vesicles [169–172].

The fifth and final phase of the bone remodeling cycle is the termination phase when mineralization of the bone matrix is fully completed. The bone enters in a quiescent state, and the amount of bone formed equals the amount resorbed. At this phase, osteoblasts undergo apoptosis, change into bone-lining cells or become buried within the newly formed bone matrix and terminally differentiate into osteocytes [140,146].

Bone remodeling is a crucial mechanism for normal bone metabolism and to maintain overall bone homeostasis. It involves a great number of cells and complex regulatory mechanisms [148,149,152–154]. Deregulation of this mechanism and involved cells is associated with numerous skeletal disorders such as osteoporosis and most importantly, RA [173,174]. Regarding this latter disease, an increased osteoclastogenesis and deregulated osteoclastic activity at bone resorption phase gives origin to bone erosions [51,175]. Therefore, osteoclasts activity, differentiation, and its regulatory mechanisms present as a key field to be studied in RA. The following topic will focus on this matter.

4.2. Altered bone metabolism in rheumatoid arthritis: bone erosion and osteoclasts

Bone erosion is one of the hallmarks of RA pathophysiology and is associated with disease severity, leading to a poor outcome and reduced quality of life [51,176]. It represents an independent risk factor for generalized osteopenia and osteoporosis [51]. The typical feature observed on plain radiographs of RA patients is the erosion of periarticular cortical bone. However, both types of bone (trabecular and cortical) are targeted for erosion in RA. Resulting from an imbalance in bone metabolism, bone erosions in RA patients are due to an excessive osteoclastic activity that leads to increased bone resorption by osteoclasts, and inadequate bone formation by osteoblasts [50,51]. In RA, bone damage and degradation are executed by osteoclasts while fibroblast-like synoviocytes are responsible for cartilage degradation [176]. Initially involving the cortical bone, the articular bone erosion leads to the destruction of the natural barrier between the surrounding tissue and the intertrabecular spaces of the bone marrow cavity [51]. Increased bone resorption activity in RA patients has been reported with osteoclasts originating from peripheral blood monocytes [130]. This means that the osteoclasts responsible for bone erosions in RA derive mainly from monocyte progenitors that infiltrate the synovial membrane [177]. In fact, the rheumatoid pannus has been shown to contain mononuclear cells exhibiting tartrate-resistant acid phosphatase (TRAP) activity, one of the markers for osteoclastic differentiation and activation [177]. The enhanced osteoclast activity in RA is due to a multiplicity of reasons that range from cellular effects to pro-inflammatory cytokines to autoantibodies [176]. MCSF, RANKL, TNF, IL-1, and IL-17 are pro-inflammatory cytokines that may play dominant roles in the pathogenesis of rheumatoid arthritis by participating in osteoclast differentiation [124].

There is evidence that joint damage and bone erosion in RA patients begins at a very early stage of the disease, progressing throughout its course. In fact, the first bone erosions perceived by

radiographic studies appear to occur during the first 2 years of the disease [178]. Both the development of erosions and the degree of radiological progression can be predicted by the rheumatoid factor (RF) and/or anti-cyclic citrullinated peptide (anti-CCP) levels at the first presentation in RA patients. This indicates that RF and anti-CCP positive patients present a higher risk for developing bone erosions with increased severity, within the first years of disease onset [179].

Many factors contribute to control the activity of osteoclasts within normal bone tissue and normal bone remodeling metabolism [148]. The complex crosstalk between the osteoclasts and neighboring cells (such as osteoblasts, osteocytes, and hematopoietic cells), along with the biochemical microenvironment, is crucial to normal osteoclastogenesis. There is evidence that certain immune complexes found in the joint of RA patients can induce macrophage activation and participate in synovial inflammation, possibly playing a role in the control of osteoclastogenesis [180,181]. Immune complexes containing anticitrullinated peptide antibodies (ACPA) may have a greater ability to stimulate osteoclastogenesis, consequently inducing an increased bone loss in RA patients [182,183]. Recent data suggest that besides being activated by immune cells, osteoclasts can play an active role in inducing or perpetuating the autoimmune feedback loops and inflammatory response in RA. They are capable of antigen presentation, which results in T cell activation [184].

5. Long non-coding RNAs (lncRNAs)

Due to the central dogma of "DNA → RNA → protein", the research of the last few decades have mainly been focused on the role of protein-coding genes in the pathogenesis of the disease. As proteins are counted as key players of cellular functions, RNA is regarded to be a mere intermediary between genes and proteins [185]. However, in recent years this paradigm has been changed. Due to the developing of new technologies, including genome and transcriptome sequencing, evidence arises suggesting that the noncoding portions of the genome may regulate the complexity of an organism [186]. In fact, the so-called "junk DNA", that comprises the noncoding portions of the genome, might significantly contribute to the higher eukaryotic sophistication, regulating gene expression, structural and functional mechanisms in cells and disease pathogenesis [187]. This concept resulted in the establishment of the Encyclopedia of DNA Elements (ENCODE) Consortium in 2003, that aimed to identify all the functional elements in the human genome [188,189]. The

ENCODE project reported that about 90% of the genome is transcribed as nonprotein-coding RNAs (ncRNAs), indicating that this might not be junk hereafter [190–192].

These transcripts that do not hold any protein-coding capacity are called non-coding RNA (ncRNA). Depending on their length, these can be classified as small non-coding RNAs (sncRNAs) and long non-coding RNAs (lncRNAs). Small non-coding RNAs are shorter than 200 nucleotides and consist mainly of microRNAs (miRNA) and small nuclear RNAs (snRNA) [193]. Long non-coding RNAs (lncRNAs) are RNAs longer than 200 nucleotides, that can be subclassified based on their intersection with protein-coding genes as intergenic or genic lncRNAs [194]. Intergenic lncRNAs do not intersect with any protein-coding loci. Genic lncRNAs comprise three biotypes: exonic lncRNA (at least one of its exons intersects a protein-coding exon by at least 1 bp), intronic lncRNAs (contained completely within a sense or antisense protein-coding introns) and overlapping lncRNAs (contain a sense or antisense coding gene within an intron) [194]. According to GENCODE v7, the lncRNAs are the most common however least well-understood RNA species [194].

The number of lncRNAs is larger than the number of protein-coding RNAs, but they are generally expressed at lower levels compared to protein-coding genes, and appear to be tissue or cell-type specific [193–195]. These RNAs can modulate gene expression through multiple mechanisms, such as epigenetics, miRNA sponging, alternative splicing, and transcriptional and translational regulation [193,196–199]. Additionally, lncRNAs appear to participate in several biological processes, such as cell proliferation, morphogenesis, pluripotency, development, neuronal processes, and gametogenesis [200]. Furthermore, the dysregulation of lncRNAs has increasingly been linked to many human diseases, especially in cancers [185,186,190,191,196,201,202].

5.1 lncRNAs in rheumatoid arthritis

Long noncoding RNAs (lncRNAs) are emerging as critical regulators of gene expression in the immune system and inflammatory responses [203,204]. These RNAs can regulate both the innate and adaptative immune responses [205–207]. Recent reports demonstrated that lncRNAs play a key role in the pathogenesis of a variety of autoimmune diseases, such as multiple sclerosis, systemic lupus erythematosus, type I diabetes and RA [208–211]. However, current knowledge of RA-related lncRNAs remains limited. The long non-coding RNA expression profile in rheumatoid arthritis patients has been mainly studied in fibroblast-like synoviocytes (FLSs) and peripheral blood mononuclear cells (PBMCs) [212–214].

An interesting analysis of the lncRNA–miRNA–mRNA functional network by Jiang *et al.,* revealed functional lncRNAs in rheumatoid arthritis, indicating that these might play important roles in the development of RA [215]. Although the biological functions and molecular mechanisms of these lncRNAs are not yet elucidated, some appear to be highly related to RA. The authors suggest that specific lncRNAs are associated with the development of RA, and highlight three lncRNAs (S5645.1, XR_006437.1, J01878) that could be used as potential diagnostic biomarkers and therapeutic targets [215]. A comprehensive analysis of lncRNA and mRNA expression profiles in rheumatoid arthritis patients was performed by Luo *et al.,* using human lncRNA microarray to analyze the PBMCs of RA patients and healthy controls [212]. A total of 5,045 lncRNA (2,410 upregulated and 2,635 downregulated) were differentially expressed in patients with RA [212].

The expression of lncRNAs and mRNAs in the PBMCs of RA patients and their role in RA pathogenesis was described by Yuan *et al.* [213]. Song *et al*., reported several lncRNAs with altered expression in PBMCs of RA patients, including Sox2OT, H19, Meg3, GAS5, NEAT1, Meg9, DANCR and HOTAIR [223]. Yang *et al.,* reported that the lncRNA *NTT* is expressed in human monocytes/macrophages and its expression is highly upregulated (around 100- to 1000-fold of normal control) in the PBMCs of early-untreated RA patients, showing a positive correlation with the initial disease activity score (DAS28) [216].

Zhang *et al*., described 62 upregulated lncRNAs and 73 downregulated lncRNAs in fibroblast-like synoviocytes from RA patients [214]. ZFAS1 is a recently described lncRNA known to be upregulated and to promote cell migration and invasion is several cancers [217–219]. Ye and its co-authors hypothesize that given these effects on cancer cells, ZFAS1 could also influence the migratory and invasive phenotypes in FLSs of rheumatoid arthritis patients [220]. In fact, they found that ZFAS1 expression is increased in synovial tissue and FLS from RA patients, and showed that silencing ZFAS1 suppressed FLS migration and invasion, while overexpression of ZFAS1 showed the opposite effect [220].

Li *et al.,* recently described a marked down-regulation of the lncRNA GAS5 in FLS of RA patients, suggesting that GAS5 down-regulation could be a pathological feature of RA [221]. GAS5 was also found to be down-regulated in both B-cells and CD4 T-cells of RA patients, although its mechanism and role in the pathophysiology of RA has not been yet elucidated [222]. One possibility is that GAS5 might participate in the regulation immune functions and pathogenesis of autoimmune and inflammatory diseases by modulating the glucocorticoid receptor (GR) transcriptional activity through its decoy RNA glucocorticoid response element (GRE) [222]. It was also reported that GAS5 expression is upregulated in PBMCs of RA patients [223].

NEAT1 is a lncRNA known to be an important regulator of the inflammatory process and highly expressed in monocytes [224]. Although there is scarce evidence of its expression in patients and role in RA pathology, it represents a putative target of interest. It is upregulated in the blood exosomes of RA patients, with a pathological relevance based in a view of delivering molecules at distances beyond that of direct cell-to-cell contact [223]. It is also upregulated in PBMCs of RA patients [223].

Recently, MEG3 was also proposed as an important lncRNA in RA pathogenesis. A research work by Liu *et al.* reported that MEG3 is significantly reduced in synovial tissue and FLSs of an RA animal model (complete Freund's adjuvant [CFA]-induced rats) [225]. It presents a methylation-dependent mechanism and regulates arthritis in this animal model by targeting the NOD-like receptor family CARD domain containing 5 (NLRC5) and regulating its expression. This, in turn, affects the proliferation of FLSs because NLRC5 promotes FLSs proliferation in RA. The enforced expression of MEG3 decreased NLRC5 expression, leading to a decreased in FLSs numbers. Loss of MEG3 in RA is because of the hypermethylation of its promoter [225].

HOTAIR is a lncRNA that has been gaining attention for its possible role in RA pathogenesis. A study by Song *et al.,* reported a notably high expression level of HOTAIR in both PBMCs and serum exosomes of RA patients, ultimately potentiating the migration of active macrophages [223]. It can function as an attractive cue for activated macrophage as well as a stimulatory cue for MMP production and activation from synovial fibroblasts. In contrast, the authors described a markedly lower level of expression of HOTAIR in *in vitro* differentiated osteoclasts and also in RA patients synoviocytes (isolated from biopsies of synovial membrane or samples of synovial fluid) [223]. Consistently, the enforced expression of HOTAIR in both osteoclasts and synoviocytes led to significantly decreased levels of MMP-2 and MMP-13 [223].

6. Aim of the study

Bone erosions in RA patients result from imbalanced bone metabolism due to excessive osteoclastogenesis and osteoclastic activity. Long non-coding RNAs (lncRNAs) have been implicated in osteoclastogenesis and RA pathophysiology. However, no study has yet examined lncRNAs expression in monocytes and osteoclasts of RA patients. This work aims to address this question by analyzing in monocytes and osteoclasts of RA patients and of healthy controls the expression of a panel of 8 lncRNAs (GAS5, NEAT1, Meg3, DANCR, HOTAIR, Meg9, H19, and Sox2OT), that have been shown to be altered during mouse osteoclastogenesis and/or altered in several cellular types in RA.

PATIENTS AND METHODS

PATIENTS AND METHODS

1. Patients and healthy controls

This study was approved by the ethics committee of Hospital de Santa Maria and all participants signed informed consent prior to enrollment. Patients with RA fulfilling the 2012 American College of Rheumatology/European League Against Rheumatism criteria [60] were recruited from the Rheumatology Department, Hospital de Santa Maria, Lisbon Academic Medical Centre, Portugal. The remaining inclusion criteria for the study groups are summarized in **Table 1.**

Heparinized blood was collected from each participant and whole blood samples were taken for isolation of peripheral blood mononuclear cells (PBMCs). Samples were stored at the Biobanco-IMM, Lisbon Academic Medical Center, Lisbon, Portugal. Age and sex-matched donors were used as healthy controls.

Table 1 - Summary of the inclusion criteria for the study groups.

Group name	Inclusion criteria
Early-untreated arthritis	< 1-year disease onset, untreated
Established rheumatoid arthritis	DAS28 > 3.2, MTX, PDN < 10mg/day
Healthy controls	Age and sex matched

DAS – disease activity score; MTX – methotrexate; PDN - prednisolone

2. Peripheral blood mononuclear cells (PBMCs) isolation

PBMCs were isolated by density gradient centrifugation using Lymphocyte Separation Medium solution (1.077 g/mL; Lonza, USA) as represented in **Figure 3**. 60 mL of blood was collected from each patient and healthy donor. Sodium heparin was used as an anticoagulant. Heparinized blood samples were diluted at 1:2 volume in a 50 mL centrifuge tube with sterile 1x Phosphate Buffer Saline (PBS) (15 mL of blood to 30 mL of PBS). Diluted blood was carefully added to Lymphocyte Separation Medium solution at 1:2 volume in a 50 mL centrifuge tube (15 mL of the solution to 30 mL of diluted blood), creating a defined layer. Samples were centrifugated for 30 min at 2000 rpm, with no brake and no acceleration, at 20 °C. This step created four distinct layers (from top to bottom): top layer containing the plasma, the layer of interest containing the PBMCs, a third layer

containing the Lymphocyte Separation Medium solution and a bottom layer with red blood cells (RBCs). The layer containing PBMCs was then carefully removed from each tube and washed two times with PBS, as follows. No more than 20 mL of PBMCs were placed in a new 50 mL centrifuge tube, and PBS was added up to a total volume of 50 mL. Samples were centrifuged for 15 min at 2000 rpm, at 20 °C. The supernatant was discarded, and the remaining pellet resuspended in 2 mL of PBS. All the resuspended pellets were collected to one of the tubes, and PBS was added up to a total volume of 50 mL. Samples were centrifuged for 10 min at 2000 rpm, at 20 °C.

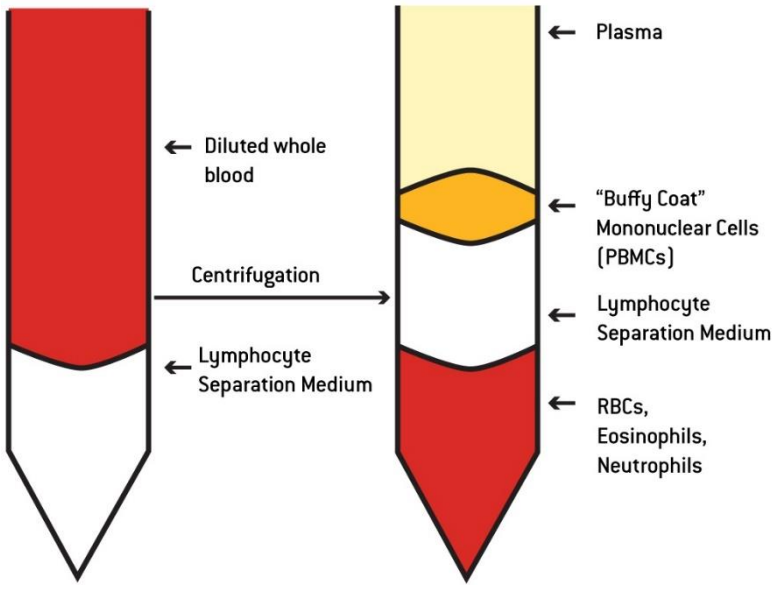

Figure 3 - PBMCs isolation with Lymphocyte Separation Medium (https://bioscience.lonza.com)

The supernatant was discarded, and the pellet was resuspended in 10 mL of PBS. Viable cells were counted on a hemocytometer, using 0.4% Trypan Blue solution (Sigma® Life Science, UK). Samples were again centrifugated for 10 min at 2000 rpm, at 20°C. The supernatant was discarded, and the cell pellet was then resuspended in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, USA) supplemented with 1% 5000 U Penicillin/Streptomycin solution (Invitrogen, USA) and 10% Fetal Bovine Serum (FBS; Invitrogen, USA), with the volume necessary to obtain the desired cell density.

3. Cell culture and osteoclastogenesis

The obtained PMBCs were seeded on plastic cell culture dishes (60.1 cm^2; Ø 87 mm; TPP®, Switzerland) at a density of 3,0 x 10^6 cells/mL in DMEM supplemented with 1% 5000 U

Penicillin/Streptomycin solution and 10% Fetal Bovine Serum (FBS). For functional assays cells were seeded on 12-well plastic cell culture dishes (3.8 cm^2; Ø 22.1 mm; Costar® Corning®, USA), on square coverslips (10 x 10 mm), at the same cell density. PBMCs were incubated in a humidified atmosphere at 37°C, 5% CO_2 and left overnight for monocytes to adhere to the plates. On the following day (day 1 of culture) selected plates with adherent monocytes were used for RNA extraction. For the remaining plates, including the 12-well plates, the *in vitro* 21-day protocol for osteoclastogenesis was performed as follows. At day 1 medium was changed to DMEM supplemented with M-CSF 25 ng/mL (Peprotech, USA) to initiate the differentiation process. Three days later (day 4 of culture), medium was changed to DMEM supplemented with M-CSF, sRANKL (50 ng/mL; Peprotech, USA), dexamethasone (10 nM; Sigma Aldrich, USA) and TGF-β (2.5 ng/mL; Peprotech, USA) to fully differentiate the osteoclast precursor cells into mature osteoclasts. The culture medium was then changed every 3 days using the same formulation until day 21 of culture. At this point, cultured mature osteoclasts were used for RNA extraction and TRAP staining.

4. Osteoclasts functional assay (TRAP staining)

Tartrate-resistant acid phosphatase (TRAP) staining of osteoclasts was performed at day 21 of culture using the Acid Phosphate Leukocyte Kit (TRAP; Sigma-Aldrich, USA) for the cytologic demonstration of the expression of acid phosphatase in mature osteoclasts. Osteoclastogenesis was performed by a 21-day differentiation protocol of adherent monocytes in square coverslips (10 mm^2). The fixative solution was prepared by combining 25 mL of citrate solution, 65 mL of acetone and 8 mL of 37% formaldehyde, placed in a glass bottle and capped tightly. 100 mL of distilled water was prewarmed at 37°C and the fixative solution was brought to room temperature (18–26°C). Cells were fixed by immersion in fixative solution for 30 seconds and then rinsed thoroughly in deionized water, never allowing coverslips to dry. In a 1 mL microtube were added 0,5 mL Fast Garnet GBC Base Solution and 0.5 mL Sodium Nitrite Solution, then mixed by gentle inversion for 30 seconds and let stand for 2 minutes. Next, a solution containing: 45 mL of deionized water prewarmed to 37°C, 1 mL of Diazotized Fast Garnet GBC Solution from the previous step, 0,5 mL of Naphthol AS-Bl Phosphate Solution, 2 mL of acetate solution and 1 mL of tartare solution was prepared and warmed to 37 °C. Coverslips were immersed in this solution and incubated for 30 min at 37°C, protected from light. After 30 min, coverslips were rinsed thoroughly in deionized water, then counterstained for 1 min in Hematoxylin Solution, Gill No. 3. Next, coverslips were rinsed for several minutes in alkaline tap water to blue nuclei and left to air dry. Finally, coverslipping was performed

with Quick-D mounting medium (Klinipath, Nederland), and slides were evaluated microscopically using an optical microscope (Leica DM2500; Leica Microsystems, Germany), for counting mature osteoclasts (TRAP+ and three or more nuclei).

5. Total RNA extraction

Total RNA was extracted from monocytes and osteoclasts with the RNeasy® Mini Kit (Qiagen, USA), using the Purification of Total RNA from Animal Cells using Spin Technology Protocol. Culture medium was aspirated, and cells were washed two times with PBS. At least 5 mL of PBS was added to each culture dish, cells were harvested, and the total volume of resuspended cells was transferred into a 15 mL or 50 mL centrifuge tube, depending on the volume obtained. Samples were centrifugated for 5 min at 2000 rpm, at 4°C. The supernatant was discarded, the pellet was resuspended in 1 mL of PBS and the suspension was transferred into a 1.5 mL microtube. Viable cells were counted on a hemocytometer, using Trypan Blue solution (Sigma® Life Science, UK). Samples were centrifuged for 5 min at 12800 rpm, at 4°C. The supernatant was discarded, and the cell pellet was loosened thoroughly by flicking the tube. Next, depending on the total number of cells, either 350 µL (< 5 x 10^6 cells) or 600 µL (5 x 10^6 – 1 x 10^7 cells) of Buffer RLT was added for lysing the pelleted cells. The lysate was homogenized by pipetting and vortexing. The protocol was usually interrupted at this step by snap freezing the samples with liquid nitrogen, and storing them at -80°C. To proceed with total RNA extraction, 1 volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. Up to 700 µL of the sample was transferred to an RNeasy spin column placed in a 2 mL collection tube. If the sample volume exceeded 700 µL, successive aliquots were centrifuged in the same RNeasy spin column, and the flow-through discarded after each centrifugation. All the following centrifugation steps were performed at 20-25°C. Samples were centrifuged for 15 s at 8000 *g* and the flow-through was discarded. Next, on-column DNase digestion with the RNase-Free DNase Set (Qiagen, USA) was performed. 350 µL of Buffer RW1 was added to the RNeasy spin column, followed by a centrifugation step for 15 s at 8000 g, to wash the spin column membrane. The flow-through was discarded. 10 µL of DNase I stock solution was added to 70 µL of Buffer RDD, mixed by gently inverting the tube, and centrifuged briefly to collect residual liquid from the sides of the tube. The DNase I incubation mix (80 µL) was added directly to the RNeasy spin column membrane, and tubes were placed on the benchtop (20–30°C) for 15 min. Next, 350 µL of Buffer RW1 was added to the RNeasy spin column. Samples were centrifuged for 15 s at 8000 *g*, and the flow-through was discarded. To proceed with

extraction, 500 µL of Buffer RPE was added to the RNeasy spin column. Samples were centrifuged for 15 s at 8000 *g*, to wash the spin column membrane. The flow-through was discarded. Another 500 µL of Buffer RPE was added to the RNeasy spin column. Samples were centrifuged for 2 min at 8000 *g*, to wash the spin column membrane. The RNeasy spin column was then placed in a new 2 ml collection tube, and the old collection tube was discarded with the flow-through. Samples were centrifuged at full speed for 1 min. This step allows the elimination of any possible carryover of Buffer RPE, or any residual flow-through remaining on the outside of the RNeasy spin column after the previous step. Then, the RNeasy spin column was placed in a new 1.5 ml collection tube, and 30 µL of RNase-free water was added directly to the spin column membrane. Samples were centrifuged for 1 min at 8000 *g*, to elute the RNA. The total RNA concentration and purity were quantified using Nanodrop 1000 (Thermo Scientific, USA). Samples were stored at -80°C.

6. Complementary DNA (cDNA) synthesis

Complementary (c)DNA was synthesized for each RNA sample at a concentration of 50 ng/µL using the NZY First-Strand cDNA Synthesis Kit, separate oligos (NZYTech, Portugal) as follows. The reaction was performed using a Tpersonal Thermal Cycler (Biometra®, Germany). The annealing reaction was performed on ice, by adding the following components into a sterile, nuclease-free microcentrifuge tube: 1 µg of RNA, 1 µL of random hexamer mix (50 ng/µL), 1 µL of 10× Annealing Buffer, and up to 8 µL of Nuclease-free water. Next, the samples were mixed gently and incubated at 65°C for 5 min. After incubation, samples were placed on ice for at least 1 min and then centrifuged briefly. On the ice, the reverse-transcription step was performed by adding 10 µL of NZYRT 2× Master Mix, no oligos, and 2 µL of NZYRT Enzyme Mix to the tube. Samples were mixed gently and centrifuged briefly, prior to incubation at 25 °C for 10 min and then at 50 °C for 30 min. The reaction was inactivated by heating at 85 °C for 5 min and then chilling the tubes on ice. Finally, 1 µL of NZY RNase H was added to each tube and samples were incubated at 37°C for 20 min. The cDNA product was stored at -20 °C until required.

7. Real-time quantitative PCR (RT-qPCR)

Each cDNA template was amplified in triplicate using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA). RT-qPCR was carried out using a ViiA 7 Real-Time PCR System (Applied Biosystems, USA). For this work, a panel of 8 lncRNAs was analyzed: GAS5, NEAT1, Meg3, DANCR,

HOTAIR, Meg9, H19, and Sox2OT (see **Table 2** for primers). For the calibration curve, cDNA was diluted in 5 different concentrations. Ten µL reactions were set up in 384-well plates with 10 µM of each primer (diluted at 1:10) and 5 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA). For RT-qPCR, 1 µL of the 20 µL reverse transcription reaction mixture was used (final cDNA concentration = 10 ng/µL). Cycling conditions were as follows: UDG activation (50°C for 2 min), Dual-Lock™ DNA polymerase (95°C for 2 min), denaturation (95°C for 15 s), annealing/extension (60°C for 1 min), repeated 40 times (40 cycles). Cycling mode was followed by a melt curve stage as follows: step 1 with ramp rate of 1.6°C/second (95°C for 15s), step 2 with ramp rate of 1.6°C/second (60°C for 1 min), dissociation at step 3 with ramp rate of 0.15°C/second (95°C for 15s). All targets were subject to melt curve analysis to test if any unspecific PCR products were generated. Amplification curves were analyzed for relative quantification using QuantStudio™ Real-Time PCR Software v1.3 analysis software (Applied Biosystems, USA). Target expression was normalized to the expression of the housekeeping gene (GAPDH) according to the comparative CT method and presented as the geometric mean of relative fold expression ($2^{-\Delta\Delta CT}$).

Table 2 - Primers used for lncRNAs expression analysis.

lncRNA	**Primer sequence**
GAPDH (housekeeping gene)	Fw 5'– GTCGTGGAGTCCACTGGCGTC – 3' Rev 5' – TCATGAGTCCTTCCACGATAC – 3'
GAS5	Fw 5'– AGAAATGCAGGCAGACCTGT – 3' Rev 5' – GCACTCTAGCTTGGGTGAGG – 3'
NEAT1	Fw 5' – GTGGCTGTTGGAGTCGGTAT – 3' Rev 5' – ATTCACTCCCCACCCTCTCT – 3'
Meg3	Fw 5' – CAGCCAAGCTTCTTGAAAGG – 3' Rev 5' – TTCCACGGAGTAGAGCGAGT – 3'
DANCR	Fw 5' – GCCACTATGTAGCGGGTTTC – 3' Rev 5' – ACCTGCGCTAAGAACTGAGG – 3'
HOTAIR	Fw 5' – GTGTAGACCCAGCCCAATTTA – 3' Rev 5' – GGCTGGACCTTTGCTTCTAT – 3'
Meg9	Fw 5' – GGCATCCCTGTACCTAGCAC – 3' Rev 5' – AGAGACAAGGCCAACAAGCA – 3'

H19	Fw 5' – TTCAAAGCCTCCACGACTCT – 3' Rev 5' – CTGAGACTCAAGGCCGTCTC – 3'
Sox2OT	Fw 5' – GCTCGTGGCTTAGGAGATTG – 3' Rev 5' – CTGGCAAAGCATGAGGAACT – 3'

8. Statistical analysis

GraphPad Prism 7.0 (GraphPad Software, USA) was used to analyze the data. The nonparametric Mann-Whitney test was used to compare the lncRNAs relative expression levels between two groups. Spearman's correlations were performed between the expression levels of the lncRNAs and clinical variables (ESR, CRP, tender and swollen joint count, and DAS28). *p*-values (two-tailed) <0.05 were considered statistically significant.

RESULTS

RESULTS

1. Patients and healthy controls

Twenty-seven subjects were initially recruited, including 8 established rheumatoid arthritis patients, 5 early arthritis patients and 14 age and gender-matched healthy donors. However, our study was only completed for 17 subjects (7 established RA patients, 3 early arthritis patients, and 7 healthy controls). As for the early arthritis patients, 2 have been diagnosed with RA and 1 still has a reserved diagnosis of rheumatic polymyalgia/seronegative RA. The clinical and demographic characteristics of the patients and healthy donors included in this study are described in **Table 3**. Early arthritis patients were all untreated. All established RA patients were on a low corticosteroid dose (prednisolone <10mg/day) and synthetic DMARDs monotherapy (4 on methotrexate and 3 with methotrexate plus hydroxychloroquine). None of the patients were on biological therapy.

Table 3 - Summary of the patients and healthy controls' characteristics.

	Healthy controls	**Early arthritis patients**	**Established RA patients**
Sample size	7	3	7
Age (years)	50 [48-62]	66 [64-78]	56 [43-65]
Females %	57%	67%	86%
Symptoms duration (years)	NA	0,4 [0,25-1]	4 [2-7]
Rheumatoid factor (% positive)	NA	67	100
ACPA (% positive)	NA	67	100
ESR (mm/h)	NA	86 [52-88]	29 [8-55]
CRP (mg/dl)	NA	1,2 [0,5-2,13]	0,56 [0,06-2,29]
DAS28	NA	5,34 [3,15-7,06]	3,76 [2,9-4,91]
Treatment	NA	none	sDMARDS

Data is represented as median [Interquartile range] unless stated otherwise; ACPA – anti-citrullinated protein antibodies; ESR – erythrocyte sedimentation rate; CRP – C-reactive protein; DAS – disease activity score; DMARDs – disease modifying antirheumatic drugs; RA – rheumatoid arthritis; NA – not applicable.

2. Cell culture, osteoclastogenesis, and total RNA extraction

The intended course of action of the study was to analyze both monocytes and osteoclasts from all study groups. However, due to technical limitations, such as lack of adequate starting number of cells (PBMCs) to isolate both monocytes and osteoclasts, it was not possible to achieve this goal. As so, the work became more focused on the collection and study of monocytes. In addition, the concentration of the obtained RNA was not high enough for reverse transcription reaction in some samples. A summary of the obtained RNA samples, suitable for cDNA synthesis and further analysis, from both monocytes and osteoclasts of all study groups, is described in **Table 4**.

Table 4 - Summary of the obtained RNA samples from both monocytes and osteoclasts of all study groups.

	Healthy controls	**Early arthritis patients**	**Established RA patients**
Monocytes	n = 5	n = 2	n = 7
Osteoclasts	n = 4	n = 2	n = 0

3. Osteoclasts functional assay (TRAP staining)

Osteoclasts (OCs) were stained for tartrate-resistant acid phosphatase (TRAP) at day 21 of culture. TRAP staining was performed to demonstrate the expression of TRAP in mature osteoclasts and thus to serve as a biochemical marker for active osteoclasts [226]. Acid phosphatase activity appears as purplish to dark red granules in the cytoplasm of mature osteoclasts **Figure 4A**. In this assay TRAP-positive cells containing three or more nuclei were counted. As shown in **Figure 4B**, *in vitro* osteoclastogenesis produced a substantially higher number of osteoclasts in early arthritis patients (1134,5±19,5 OCs/mm^2 [mean±SD]), when compared to healthy controls (279,25±140,30 OCs/mm^2 [mean±SD]). As the same starting number of cells was used (3,0 x 10^6 cells/mL) in all samples, these results suggest that peripheral monocytes of early arthritis patients are more prone to osteoclastogenesis.

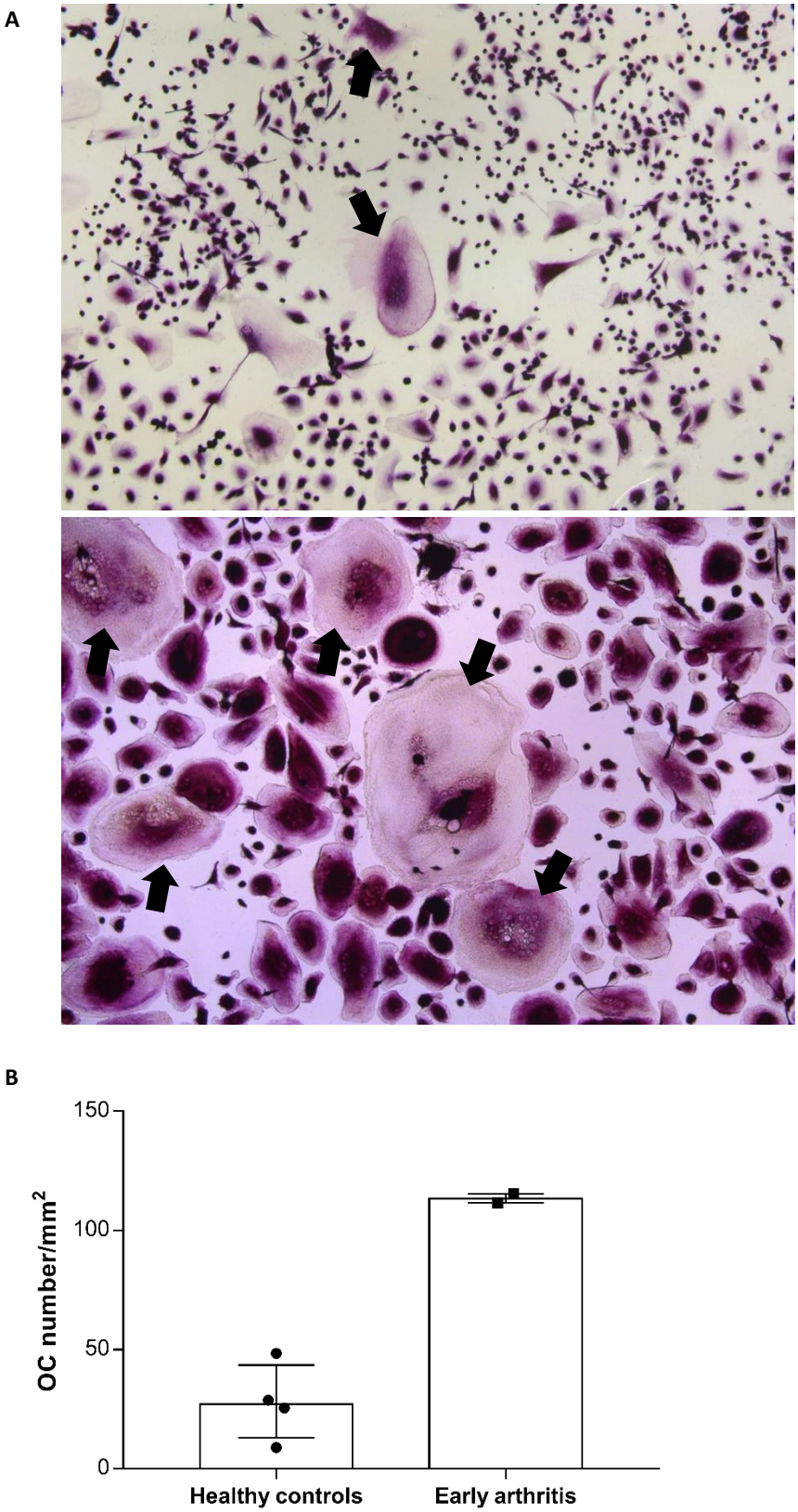

Figure 4 - TRAP staining of in vitro differentiated osteoclasts. A: representative image of OCs of healthy controls (top) and early arthritis patients (bottom) (10× magnification) at culture day 21, stained for TRAP. Black arrows – osteoclasts. B: OC number were increased in early arthritis patients (n=2), when compared to healthy controls (n=4). Each dot represents a sample. Data presented as median with interquartile range.

4. lncRNAs expression analysis by RT-qPCR

A panel of 8 lncRNAs was analyzed (GAS5, NEAT1, Meg3, DANCR, HOTAIR, Meg9, H19, and Sox2OT) in both monocytes and osteoclasts from healthy controls (n=5 and n=4, respectively), early arthritis patients (n=2 and n=2, respectively) and in monocytes from established rheumatoid arthritis patients (n=7). From these, measurable and reproducible expression was only observed for 3 lncRNAs (GAS5, NEAT1, and DANCR) by RT-qPCR in all samples of both monocytes and osteoclasts, of all the study groups. As so, the results herein presented refer only to the expression levels of GAS5, NEAT1, and DANCR.

As shown in **Figure 5**, NEAT1 presents an increased expression in monocytes of both early arthritis patients and established RA patients, when compared to monocytes of healthy controls. GAS5 appears to have an increased expression in monocytes of established RA patients, and a decreased expression in monocytes of early arthritis patients, when compared with those of healthy controls. DANCR shows a slight decrease in expression in monocytes of established RA patients and no differences in early arthritis patients when compared to healthy controls. However, no statistically significant differences were found between monocytes of healthy controls and established RA patients, in either of lncRNAs.

The expression of these lncRNAs was also analyzed in osteoclasts of early arthritis patients and healthy controls **Figure 6**. NEAT1 presents an increased expression in osteoclasts of early arthritis patients when compared to those of healthy controls. Regarding GAS5 and DANCR, they both show a slightly decreased expression in osteoclasts of early arthritis patients, when compared to those of healthy controls.

A comparison of the relative expression of these lncRNAs between monocytes and osteoclasts was performed for both healthy controls and early arthritis patients, and it is presented in **Figure 7** and **Figure 8**, respectively. In healthy controls, osteoclasts have an increased expression of both NEAT1, GAS5, and DANCR, when compared to monocytes. However, no statistically significant differences were found between monocytes and osteoclasts of healthy controls, in either of lncRNAs.

Regarding early arthritis patients, the same tendency of increased expression in osteoclasts is observed for GAS5 and DANCR. In contrast, NEAT1 expression is decreased is osteoclasts of early arthritis patients, when compared to monocytes.

For the remaining lncRNAs, no measurable expression was detected for all samples in all groups (data not shown). As primer optimization was performed in PMBC RNA samples, we can conclude that both monocytes and osteoclasts do not express these lncRNAs in all the experimental groups tested.

On the following table (**Table 5**) we present the values for relative expression of NEAT1, GAS5, and DANCR, along with median and interquartile range. The expression was normalized for the housekeeping gene (GAPDH) expression values. Some values were excluded (*) for the analysis, based on high discrepancy relative to the remaining values of the group.

Table 5 - Relative expression values for NEAT1, GAS5, and DANCR.

	Monocytes HC	**Monocytes RA**	**Monocytes EA**	**Osteoclasts HC**	**Osteoclasts EA**
NEAT1	*4,469** 0,46 0,571 0,824 1,035	1,306 1,238 1,002 0,804 1,238 *0,034** 1,27	2,533 2,128	2,358 0,434 0,976	1,299 1,558
Median [IQ range]	0,6975 [0,46-1,035]	1,238 [0,804-1,306]	2,3305 [2,533-2,128]	0,976 [0,434-0,976]	1,4285 [1,299-1,558]
GAS5	*4,441** 0,26 0,835 0,52 1,995	0,66 0,288 2,852 1,319 1,009 0,259 *5,558**	0,331 0,585	1,494 0,691 0,968	0,75 0,849
Median [IQ range]	0,6775 [0,26-1,995]	0,8345 [0,259-2,852]	0,458 [0,331-0,585]	0,968 [1,494-0,691]	0,7995 [0,75-0,849]
DANCR	*3,728** 0,493 0,911 0,728 0,82	0,719 0,652 1,31 0,555 0,661 0,226 *3,316**	0,797 0,876	0,75 1,198 1,113	0,892 1,037
Median [IQ range]	0,774 [0,493-0,911]	0,6565 [0,226-1,31]	0,8365 [0,797-0,876]	1,113 [0,75-1,198]	0,9645 [0,892-1,037]

**excluded values* – based on discrepancy within the group

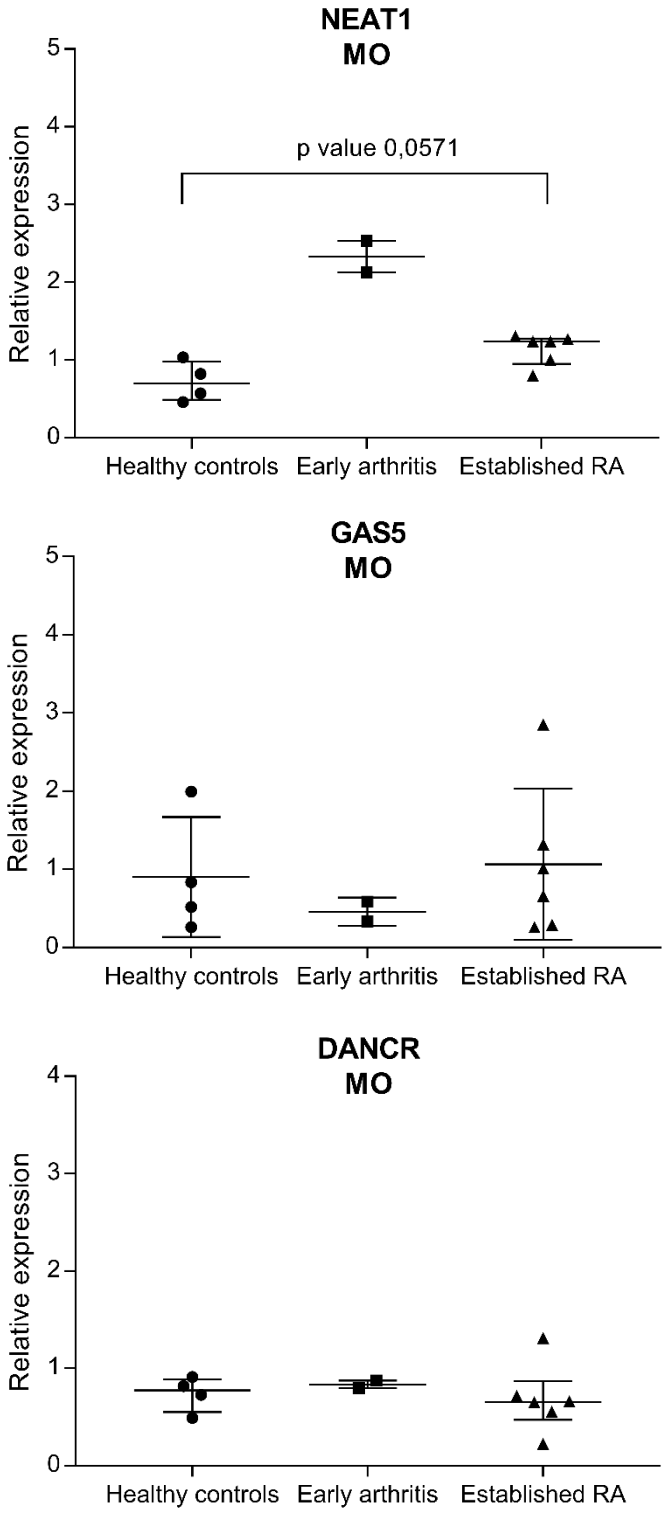

Figure 5 - Relative expression of NEAT1, GAS5 and DANCR in monocytes from healthy controls (n=4), early arthritis patients (n=2) and established rheumatoid arthritis patients (n=6). Data was normalized for housekeeping gene (GADPH) expression levels. No statistically significant differences were found between healthy controls and established RA patients. Each dot represents a sample. Data presented as median with interquartile range. RA – rheumatoid arthritis; MO – monocytes.

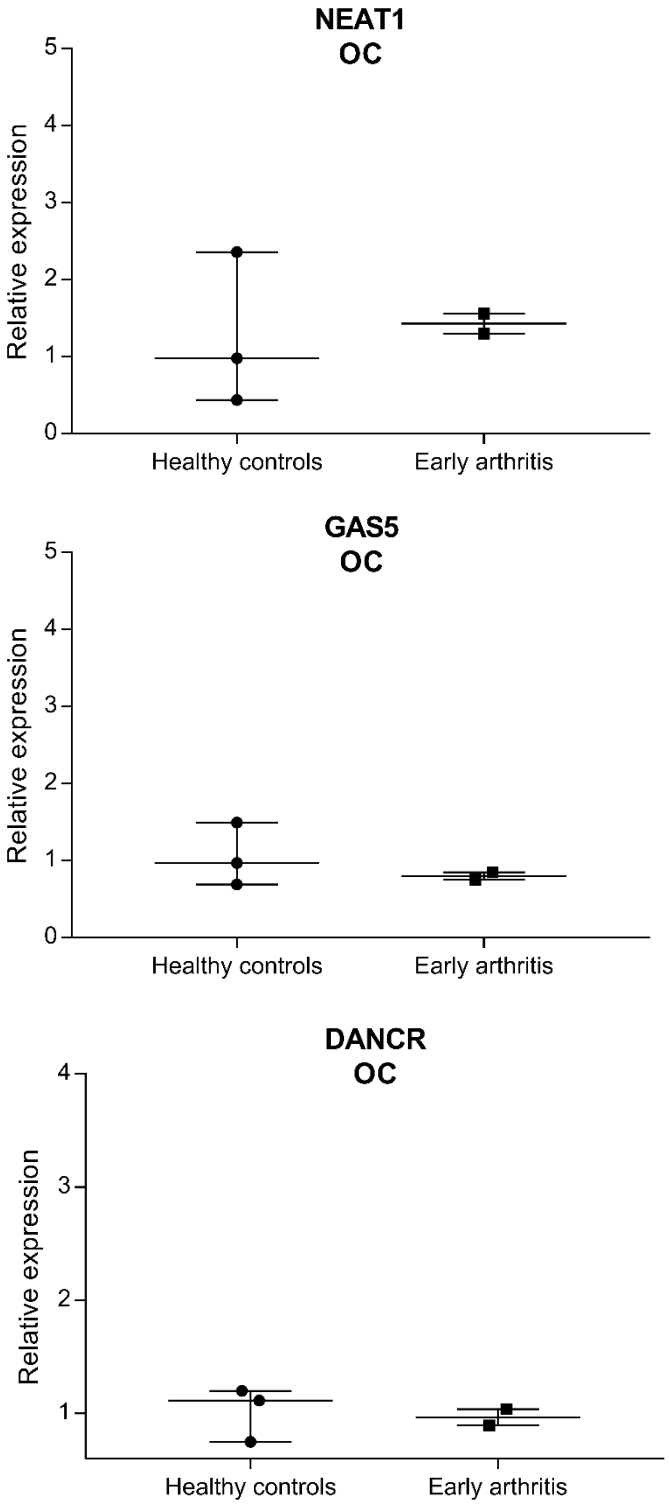

Figure 6 - Relative expression of NEAT1, GAS5 and DANCR in osteoclasts from healthy controls (n=3), and early arthritis patients (n=2). Data was normalized for housekeeping gene (GADPH) expression levels. No statistical analysis was performed due to insufficient number of samples. Each dot represents a sample. Data presented as median with interquartile range. OC – osteoclasts.

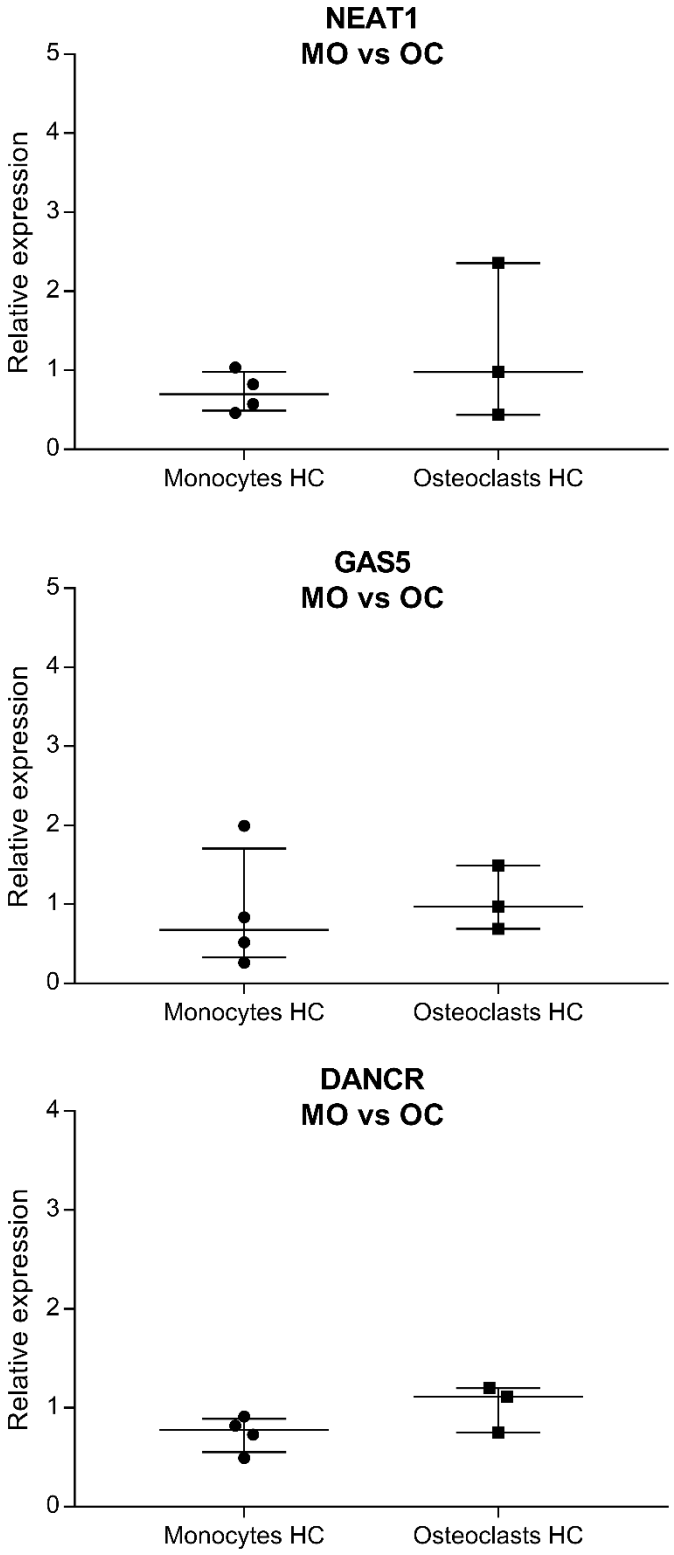

Figure 7 - Relative expression of NEAT1, GAS5 and DANCR in monocytes (n=4) and osteoclasts (n=3) from healthy controls. Data was normalized for housekeeping gene (GADPH) expression levels. No statistically significant differences were found between monocytes and osteoclasts of healthy controls. Each dot represents a sample. Data presented as median with interquartile range. HC – healthy control; MO – monocytes; OC – osteoclasts.

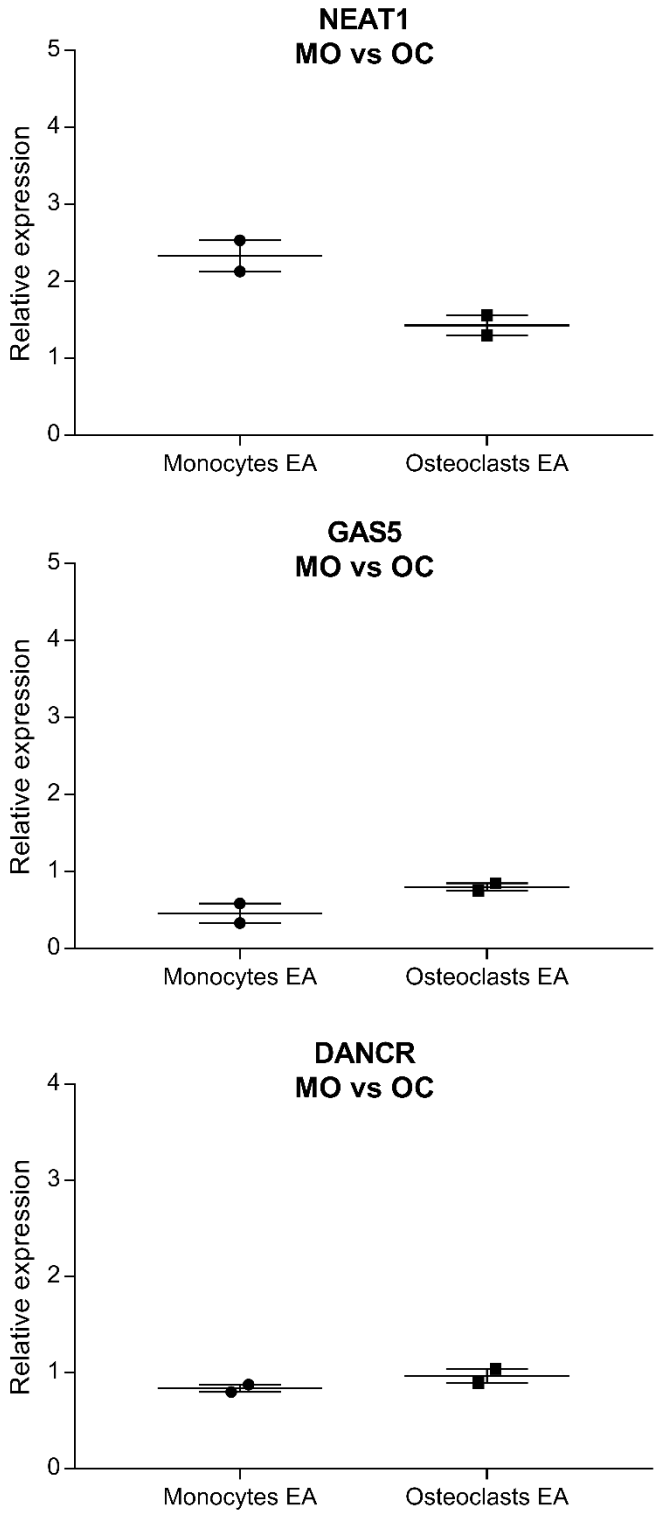

Figure 8 - Relative expression of NEAT1, GAS5 and DANCR in monocytes (n=2) and osteoclasts (n=2) from early arthritis patients. Data was normalized for housekeeping gene (GADPH) expression levels. No statistical analysis was performed due to insufficient number of samples. Each dot represents a sample. Data presented as median with interquartile range. EA – early arthritis; MO – monocytes; OC – osteoclasts.

5. Correlation of lncRNAs expression and clinical variables

A Spearman correlation analysis of the expression levels of NEAT1, GAS5 and DANCR in monocytes of established RA patients was performed for the following clinical variables: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), number of tender joints, number of swollen joints and disease activity score (DAS28). The results are summarized in **Table 6**.

In established RA patients, GAS5 expression in monocytes was significantly negatively correlated with the number of tender joints (r= -0.9063, p= 0.0095). Data are represented in **Figure 9** along with the linear regression analysis obtained for this correlation.

Table 6 - Spearman correlation analysis of lncRNAs expression and clinical variables of RA patients.

	NEAT1		**GAS5**		**DANCR**	
	r	p value	r	p value	r	p value
ESR	-0,1081	0,8206	0,2143	0,6615	0,1786	0,7131
CPR	0,02727	0,9599	0,4505	0,3111	0,5586	0,2056
Tender joints	-0,169	0,7143	-0,9063	0,0095 **	-0,5714	0,2000
Swollen joints	-0,01888	0,9952	-0,2433	0,5952	0,05614	0,9238
DAS28	-0,2342	0,6198	-0,1786	0,7131	-0,03571	0,9635

** significance considered for p value < 0,05

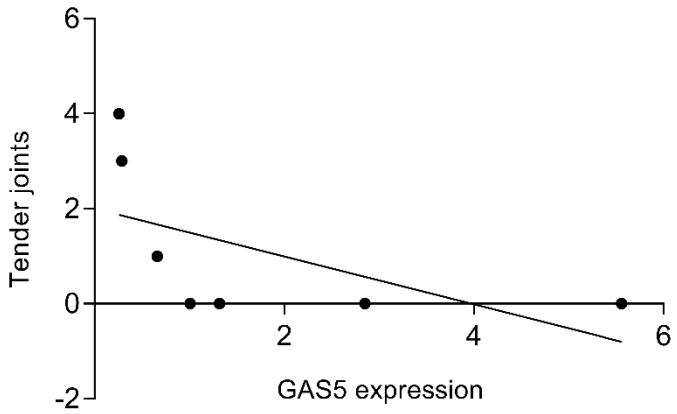

Figure 9 - Correlation analysis between GAS5 expression and the number of tender joints in RA patients. Line represents linear regression obtained for these variables: Y = -0,5045*X + 2,004; R^2 = 0,3319.

DISCUSSION

DISCUSSION

All in all, rheumatoid arthritis can be characterized by synovial inflammation and hyperplasia ("swelling") in joints, autoantibody production, cartilage and bone destruction, and systemic manifestations [34]. Bone erosion is a crucial hallmark of RA pathophysiology and is associated with disease severity [51]. There is evidence that bone erosions start to occur within the first year of disease onset, representing one of the first and most important features of RA pathogenesis [178,179]. These erosions in RA patients result from an imbalanced bone metabolism due, in part, to excessive osteoclastogenesis and osteoclastic activity, that leads to increased bone resorption by osteoclasts [50–52]. Besides being responsible for bone resorption, osteoclasts are key players in the complex cross-talk between bone and immune cells, that leads to the perpetuation of the inflammatory environment in joints of RA patients [53–55].

Once established, bone erosions rarely repair. Current biological and synthetic DMARDs therapeutic can control inflammation in RA, achieving clinical remission or low disease activity, halt the erosive process and lead to partial repair of bone erosions. However, up until now, these drugs cannot induce full repair and regression of the erosions [51,175].

It is then imperative to find new, more effective, and targeted therapeutic approaches to osteoclasts and osteoclastogenesis, to prevent bone erosion in RA patients and control the inflammatory cascades. For this, we must first understand the pathophysiological mechanisms underlying the excessive osteoclastogenesis and osteoclastic activity present in RA. In this regard, the altered expression of long non-coding RNAs (lncRNAs) has been observed during mouse osteoclastogenesis, suggesting a physiologic role for lncRNAs in the process [227]. Moreover, in RA patients, lncRNA expression has been shown to be altered in cellular types critical for its pathophysiology, like peripheral blood mononuclear leukocytes and activated fibroblast-like synoviocytes, in comparison to healthy controls [79,213,214]. However, no study has yet examined lncRNAs expression in monocytes and osteoclasts of RA patients. Taken this, this research work was designed to address this issue and gain further insight on the role of lncRNAs in the cellular mechanisms controlling the function of osteoclasts and monocytes, specifically in early arthritis and established RA patients.

To tackle this, an *in vitro* differentiation protocol of osteoclasts was performed using monocytes isolated from early arthritis patients and healthy controls. As described in section 3 of the results, we observed a 4-fold increase in the number of fully differentiated osteoclasts in early arthritis

patients, when compared to those of healthy controls. These results suggest that peripheral monocytes of early arthritis patients appear to be more prone to osteoclastogenesis, which is consistent with the increased differentiation of osteoclasts and the imbalance in bone metabolism usually documented in RA patients [130]. In this regard, it has been reported that RA patients present a higher percentage of $CD14^+$ osteoclast precursors in the blood circulation in comparison to healthy controls and that in vitro-derived osteoclasts present lower apoptosis rates than those derived from the control group [130]. Altogether, these results suggest that these two factors might contribute to the larger number of osteoclasts found in RA patients. Other authors have further reported an increased monocyte turnover, along with a reduced circulation time in the blood and a most prominent activation of monocytes in the joints of RA patients [228]. These findings indicate that there is an increased number of active monocytes in RA, that ultimately leads to increased osteoclastogenesis. In addition, the increased bone resorption by osteoclasts that leads to bone erosions in rheumatoid arthritis patients might also be due to increased functional activity, and not only to increased numbers of circulating precursors [229].

It is known that in the inflamed RA joint the formation of immune complexes, in parallel with rheumatoid factor production, stimulates monocytes and macrophages to produce TNF-α [180]. The presence of this and other pro-inflammatory cytokines is known to stimulate osteoclast differentiation and activity, and thus, to increase bone resorption in RA [230]. RANKL, a critical cytokine in osteoclastogenesis is expressed by both synovial fibroblasts and activated T cells among other cell types present in synovial tissues from RA patients [231]. These synovial cells thus contribute directly to the expansion of osteoclast precursors and to the formation and activation of osteoclasts at sites of bone erosion in RA. As so, the synovial tissue in RA is a source of osteoclasts differentiation factors and pro-inflammatory cytokines that foster increased osteoclastogenesis and consequently, an increased osteoclastic activity, that ultimately lead to bone erosions in RA patients [230,231].

After obtaining fully differentiated osteoclasts from both early arthritis patients and healthy controls, the expression of a panel of lncRNAs was analyzed. From this panel, only NEAT1, GAS5, and DANCR were shown to be expressed in the in vitro-derived osteoclasts from both groups. Our results showed that these lncRNAs are differentially expressed in osteoclasts of early arthritis patients when compared to those of healthy controls (Figure 5). In particular, we observed an increased NEAT1 expression and a slightly decreased expression for both GAS5 and DANCR in osteoclasts of early arthritis patients when compared to healthy controls. Despite the fact that no meaningful statistical analysis could be performed in view of the reduced number of samples

collected, our preliminary data suggest to us that the assayed lncRNAs may play a role in the increased osteoclastogenesis and/or enhanced osteoclastic activity reported in RA patients.

Such a proposal is in accordance with previously published studies. A recent report by Cui *et al.,* using a mice model (C57BL/10 mice) suggested that EPC (endothelial progenitor cell) - derived exosomes could promote osteoclastogenesis through the lncRNA-MALAT1/miR124 pathway. In this report, lncRNA-MALAT1 was shown to directly bind to miR-124 and subsequently, to negatively control the microRNA activity. Moreover, miR-124 overexpression was observed to reverse the migration and osteoclastic differentiation of bone marrow-derived macrophages induced by EPC-derived exosomes [232]. On the other hand, a study by Dou *et al.,* using RAW264.7 mouse cells as osteoclasts precursors for *in vitro* osteoclastogenesis, reported that most RANKL-dependent gene expression was upregulated during osteoclastogenesis and that specific gene expression changes were observed at different stages of differentiation [227]. Importantly, the authors profiled the expression of lncRNAs, by microarray, during mouse osteoclastogenesis. In comparison to unstimulated RAW264.7 cells, they found 1,643 lncRNAs upregulated and 2,705 lncRNAs downregulated in pre-osteoclasts. In mature osteoclasts, 1,896 lncRNAs were upregulated and 2,706 lncRNAs were downregulated. In activated osteoclasts, 2,716 lncRNAs were upregulated and 3,124 lncRNAs were downregulated [227]. Overall, they reported that at distinct stages of osteoclastogenesis, thousands of lncRNAs were differentially expressed compared to the control group. A different study with RAW264.7 mouse cells reported that lncRNA-Jak3 and Jak3 co-expression patterns were significantly upregulated in differentiated osteoclasts, inducing the upregulation of the expression of cathepsin K [233]. Finally, it was recently reported by Liu *et al.,* that the expression of the lncRNA AK077216 is significantly upregulated during mouse osteoclastogenesis, and that the up and downregulation of this lncRNA, respectively, promotes and inhibits osteoclast differentiation, bone resorption, and the expression of TRAP related genes [234]. These pieces of evidence further support the hypothesis that lncRNAs play a critical role in the differentiation and activation of osteoclasts.

As previously described, monocytes play a key role in modulating the pathophysiological mechanisms of RA, besides being the precursor cells for osteoclasts. Therefore, one of the focus of this study was to analyze the expression of the selected panel of lncRNAs in these cells. Again, as previously with osteoclasts, we only observed the expression of NEAT1, GAS5, and DANCR in monocytes of all groups studied. For these lncRNAs, we observed their differential expression in monocytes of early arthritis patients and established RA patients when compared to those of healthy controls (Figure 4). In particular, monocytes of established RA patients displayed an

increased expression of NEAT1, a slightly increased expression of GAS5 and a minor decreased expression of DANCR, when compared to those of healthy controls. Regarding the monocytes of early arthritis patients, we observed an increased expression of NEAT1, a decreased expression of GAS5 and no relevant expression variation for DANCR, when compared to healthy controls. Despite the fact that the observed expression variations were not statistically significant, these results suggest to us that these particular lncRNAs could play a role in monocyte biology in RA, perhaps, contributing to the increased number of blood circulating monocytes and/or to the enhanced monocyte proinflammatory and immunomodulatory activities reported in RA patients.

LncRNAs have been recently predicted to constitute important regulators of the human innate immune response, and in particular, of the function of human monocytes [235]. But, although there are some publications describing the profile of lncRNAs expression in human monocytes [236,237], their functional role in these cells remains largely unclear. A recent study by Riege *et al.* concluded that lncRNA expression is affected in human monocytes in response to bacterial and fungal infections and that some lncRNAs stimulate the activation of specific immunomodulatory activity in monocytes [238]. Moreover, evidence indicates that the abnormal expression of lncRNAs in monocyte-derived dendritic cells may be involved in the pathological processes of systemic lupus erythematosus (SLE), with potential value for the assessment of disease activity in SLE accordingly to levels of expression of some lncRNAs [239].

When considering our data on lncRNA expression, differential expression of NEAT1, GAS5 and DANCR were further detected between monocytes and osteoclasts of both healthy controls and early arthritis patients (Figures 6 and 7). In healthy controls, the expression of all 3 lncRNAs was increased in osteoclasts, when compared to monocytes. Regarding early arthritis patients, we observed a decreased expression of NEAT1 and a tendency for increased expression of both GAS5 and DANCR. Although a meaningful statistical analysis could not be performed taken the reduced number of samples analyzed for osteoclasts, our results collectively suggested to us that these lncRNAs can possibly play a role in the osteoclastogenesis process, in both physiological and pathological settings. This proposal is in agreement with the previously mentioned study by Dou et al. where a differential expression of several lncRNAs was reported during different time points of mouse osteoclastogenesis [227].

In the present report, we have observed that NEAT1 expression was upregulated in both monocytes and osteoclasts of early arthritis patients and established RA patients when compared to healthy controls. As far as we know, our study is the first to report similar findings. In fact, until now, no published work has particularly analyzed the expression of NEAT1 in monocytes and osteoclasts, in

the context of rheumatoid arthritis patients. Still, a recent study by Shui *et al.* reported a significant upregulation of NEAT1 in PBMCs of RA patients, which are in accordance with our findings [240]. The authors have further performed an *in vitro* differentiation of $CD4^+$ T cells isolated from RA patients into Th17 cells and showed that NEAT1 was also upregulated in these Th17 cells [240]. These are proinflammatory T cells with a critical role in RA pathogenesis, as they produce IL-17, one of the most important proinflammatory cytokines in RA [241]. Moreover, knockdown of NEAT1 positively inhibited Th17/$CD4^+$ T cell differentiation through reducing the signal transducer and activator of transcription 3 (STAT3) protein level [240]. In such a way, lncRNA NEAT1 should play an important role in RA pathogenesis and should, therefore, be considered as a potential therapeutical target for RA treatment [240]. In addition, NEAT1 was shown to be predominantly expressed in human monocytes and importantly, its expression was shown to be abnormally increased in both PBMCs and monocytes of systemic lupus erythematosus (SLE) patients [224]. Furthermore, Zhang *et al*. reported a positive correlation between NEAT1 expression levels and clinical disease activity in SLE patients [224]. Overall, the increased NEAT1 expression could be a potential contributor to the elevated production of several cytokines and chemokines in SLE patients [224].

Although the pieces of evidence are still scarce, these studies clearly suggest a crucial role for NEAT1 in regulating the inflammatory processes in autoimmune diseases such as RA and SLE. Beyond this, other functions have been proposed for this lncRNA in human diseases. Namely, NEAT1 has been shown to: (i) promote the proliferation and metastasis in breast cancer [242]; (ii) promote tumorigenesis in colorectal cancer [243]; (iii) predict various malignant tumor lymph node metastasis [244] and (iv) correlates with increased disease risk, elevated severity, and unfavorable prognosis as well as higher expression of pro-inflammatory cytokines in sepsis patients [245].

Concerning GAS5 lncRNA, when compared to healthy controls, our results showed that its expression is downregulated in both monocytes and osteoclasts of early arthritis patients and slightly upregulated in monocytes of established RA patients. To our knowledge, the current study is the first to report these findings. Until now, no published reports have investigated the expression of GAS5 in monocytes and osteoclasts, in the context of rheumatoid arthritis patients. However, a recent study by Li *et al.* reported that GAS5 expression is significantly decreased in the synovial tissues and fibroblast-like synoviocytes (FLSs) of RA patients, suggesting that GAS5 downregulation could be a pathological feature of RA probably by inhibiting FLSs apoptosis [221]. Through a series of experiments, the authors hypothesized that GAS5 overexpression could be a viable tool for inhibiting FLSs proliferation in RA [221]. These results could support our findings since early arthritis patients are non-treated and represent the first and often most severe presentation of the disease,

with the downregulation of GAS5. Additionally, GAS5 expression in monocytes of established RA patients was observed to be significantly negatively correlated with the number of tender joints in these patients. This result would suggest that a reduction in the number of tender joints in RA patients is associated with a higher expression of GAS5 in monocytes. However, despite the obtained statistical significance, a closer inspection of the distribution of the data (Figure 8) allowed us to understand that most of the data are highly deviated from the linear regression line obtained with them, i.e., that this is not a robust correlation. Still, we propose that the slight upregulation of GAS5 in the monocytes of RA patients could be related with a decreased degree of inflammation in RA joints, suggesting a role for GAS5 as a modulator of the immunoinflammatory pathways in RA.

Moreover, it was reported by Song *et al.* that GAS5 is upregulated in chondrocytes of osteoarthritis (OA) patients [246]. This upregulation was found to be associated with increased activation of several MMPs, suggesting that GAS5 plays a role in OA pathogenesis [246]. Another study by Guo *et al.* reported that GAS5 acts as a tumor suppressor lncRNA in endometrial cancer, by significantly enhancing the expression of PTEN to promote cancer cell apoptosis [247]. Data published by Sugatani *et al.* demonstrated multiple roles for PTEN in RANKL-induced osteoclast differentiation and OPN-stimulated cell migration, in RAW 264.7 osteoclast precursors [248]. The authors showed that PTEN overexpression suppressed RANKL-mediated osteoclast differentiation and OPN stimulated cell migration [248]. Based on these pieces of evidence we can propose that GAS5 could play important roles in RA pathogenesis, on one hand, by modulating FLSs survival and migration, and on the other, by modulating osteoclast differentiation and migration. Through these effects, GAS5 may critically contribute to cartilage and bone destruction, typically observed in RA.

Beyond rheumatic disease, GAS5 has been observed to be implicated in other pathologies. Importantly, this lncRNA has been reported to be implicated and aberrantly expressed in a variety of human cancers. In a recently published study by Cheng *et al.,* reduced GAS5 levels were significantly associated with advanced clinical stage and lymph node metastasis in colorectal cancer (CRC) patients [249]. Additionally, GAS5 overexpression suppressed CRC cell proliferation and promoted cellular apoptosis [249]. Accordingly, several findings indicate that GAS5 expression is decreased in other cancers and that its overexpression functions as a tumor suppressor in prostate cancer [250], osteosarcoma [251] and ovarian cancer [252].

Regarding DANCR, our results showed that this lncRNA expression is slightly downregulated in both monocytes of established RA patients and osteoclasts of early arthritis patients when compared to healthy controls. Interestingly, no differences in DANCR expression were found in monocytes of early arthritis patients and healthy controls. To our knowledge, this study is the first to report these

findings. Until now, there are no published reports where the expression of DANCR was thoroughly inspected in monocytes and osteoclasts, in the context of rheumatoid arthritis patients. This lncRNA has instead been implicated in the pathogenesis of osteoarthritis. A study by Xiao *et al.* recently reported that DANCR is differently expressed in human knee osteoarthritis patients [253]. In addition, Zhang *et al.* showed that the expression of DANCR was significantly increased in OA patients, and further implicated this lncRNA in promoting the proliferation, inflammation, and reduced cell apoptosis of chondrocytes in OA [254]. Consistently, similar results were reported by Fan *et al.* [255]. These pieces of evidence place DANCR as a critical therapeutic target to tackle OA and moreover, as a potential mediator in the pathological mechanisms underlying other chronic joint diseases, such as RA. Interestingly, however, in contrast to our results, an upregulation of DANCR in the blood monocytes of postmenopausal women with increased risk for osteoporosis was reported by Tong *et al.* to be correlated with increased levels of IL-6 and TNF-α in these patients. These results led the authors to suggest that DANCR could be involved in the pathology of osteoporosis [256].

Beyond the scope of rheumatology, several recently published studies have highlighted the role of DANCR in promoting tumor progression [257–260]. Gao *et al.* reported that ovarian cancers displayed a significantly higher expression of DANCR in comparison to that detected in the corresponding normal tissues [257]. Upon DANCR overexpression, the authors have importantly shown that the proliferation, invasion, and migration of ovarian cancer cells were markedly increased [257]. Similar findings were reported in a study by Tao *et al.* in breast cancer patients: (i) breast cancer samples displayed a similar upregulation of DANCR expression and (ii) higher DANCR expression levels were correlated to a poorer prognosis [258].

As mentioned in the Results section and contrary to our expectations, we did not detect the expression of Meg3, Meg9, HOTAIR, H19, and Sox2OT lncRNAs, in both monocytes and osteoclasts of early arthritis and RA patients, and healthy controls. Still, the expression of these lncRNAs was measurable in PBMCs of healthy controls (data not shown). From these results, our data on HOTAIR expression were the most unexpected ones taken that this lncRNA was previously reported to be expressed in differentiated osteoclasts of RA patients [223]. This data discrepancy may be due to the use of distinct differentiation protocols for *in vitro* osteoclastogenesis.

The role of lncRNAs in RA pathogenesis was explored by Jiang *et al.,* in a study that aimed to identify the expressed lncRNAs that could be relevant to adjuvant-induced arthritis (AA) in rats and to explore the possible molecular mechanisms of RA pathogenesis [261]. The results revealed that multiple clusters of lncRNAs were uniquely expressed in AA rats compared with controls, which

implies that these differentially expressed lncRNAs in AA rats might have a vital role in RA development [261].

Summarizing, increasing evidence indicates that lncRNAs function as key modulators of the immunoinflammatory pathways in RA, as well as of pathological mechanisms of monocytes and osteoclasts, suggesting that they could be putative therapeutic targets. Dysregulation of the expression levels of several lncRNAs has been pinpointed as possible components of the pathological mechanisms underlying rheumatoid arthritis [79,212–214,262,263]. The present research work contributes to this field by providing the first data on the expression of specific lncRNAs in both monocytes and osteoclasts of early arthritis and established RA patients. Nevertheless, further studies will be needed to fully understand the involvement of lncRNAs, as well as, their specific mechanisms of action in RA pathogenesis.

FINAL REMARKS

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In the past decade, long non-coding RNAs have emerged as important players in gene regulation and in an increasing diversity of scientific areas, including, disease pathogenesis. In this regard, research on lncRNAs represents nowadays a new frontier in the study of human diseases [185–187].

In this study, we present preliminary data that suggest, for the first time, differential expression of several lncRNAs in monocytes and osteoclasts of rheumatoid arthritis patients. Regarding NEAT1, our results are suggestive of an upregulation of this lncRNA in early arthritis and established RA, leading us to propose NEAT1 to be closely associated with rheumatoid arthritis pathogenesis, most probably as a key modulator of immunoinflammatory processes in RA. In what concerns GAS5, our results led us to propose that the slight upregulation of this lncRNA observed in the monocytes of RA patients could be related with a decreased degree of inflammation in RA joints, suggesting a role for GAS5 as a modulator of the immunoinflammatory pathways in RA. Finally, a slight downregulation of DANCR was observed in both monocytes of established RA patients and osteoclasts of early arthritis patients. Altogether, these results led us to suggest that DANCR may possibly play a role in the pathological mechanism of chronic joint inflammation in RA.

Inflammatory bone resorption mediated by osteoclasts is a major cause of morbidity and disability in many inflammatory disorders, including rheumatoid arthritis. The mechanisms that regulate osteoclastogenesis and bone resorption in inflammatory settings are complex and have not been fully elucidated [264]. With this study, we provide preliminary data that collectively suggest that the studied lncRNAs might play a significant role in the altered bone metabolism present in RA patients, perhaps by modulating immunoinflammatory pathways that impact on chronic joint inflammation and bone erosion. Although a limited study on the role of lncRNA in rheumatoid arthritis is available, an increasing number of authors recognize the potential of these RNAs in RA pathogenesis [79,261–263]. In fact, it is already known that long noncoding RNAs are involved in gene regulation in the immune system [203] and the transcriptional control of inflammatory responses [204]. Moreover, lncRNAs participate in the regulation of both the innate and adaptative immune responses [205–207]. Additionally, these RNAs have been pinpointed as key players and potential therapeutic targets in autoimmune diseases [209].

As future perspectives, we highlight the need for further research work on the functional roles of lncRNAs in the pathogenesis of rheumatoid arthritis. A major limitation of our study concerns the

reduced number of samples collected that may have precluded us from obtaining statistically significant results. To circumvent this, a larger cohort of RA patients should obviously be sampled in the future. In addition, this study may need some strategic adjustments, some of which have already started to be implemented during the course of the current work. Our initial aim was to analyze lncRNA expression in both monocytes and osteoclasts of all study groups. However, along the way, we realized that despite collecting a significant amount of blood, it was not possible to isolate enough monocytes to simultaneously collect a sufficient amount of total RNA for studying lncRNA expression in these cells and also differentiate them into osteoclasts. To circumvent this, we figured that this expression study could be performed in separate moments for monocytes and osteoclasts. Thereof and for improving sample collection, we concentrated our attention on addressing lncRNA expression in monocytes. Alternatively, to overcome the limitation of the reduced amount of total RNA collected for some samples, one hypothesis may also be to reduce the amount of total RNA used for cDNA synthesis. A different approach to bypass these problems in the future may as well be to employ a transcriptomic analysis for lncRNA expression such as, with microarrays or RNA sequencing. When in place, these strategies will also provide us with a full and comprehensive analysis of lncRNA expression in monocytes and osteoclasts in RA.

Still, the current work provided a first study of the expression of lncRNAs in monocytes and osteoclasts of RA patients that will pave the way for understanding the role of these RNAs in RA. Such insight will be pivotal for fully exploring the vast potential use of lncRNAs as therapeutic targets in RA and other immune-mediated diseases.

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